EFFECTS OF FOLLICULAR AGING AND DURATION OF SUPERSTIMULATION ON OOCYTE COMPETENCE AND GRANULOSA CELL GENE EXPRESSION IN CATTLE

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ABSTRACT

A prolonged growth phase of the ovulatory follicle results in follicular aging. Whether follicular aging is detrimental or beneficial to oocyte competence is not fully known. The objective of this thesis is to investigate the effects of follicular aging on oocyte competence and granulosa cell gene expression in cattle. Four sets of experiments were designed to address the objective. The following hypotheses were tested during the course of these studies: 1) oocyte competence will improve by the longer growing phase but will be adversely affected by FSH starvation, 2) follicles that undergo superstimulation will have different gene expression than dominant follicles from a natural cycle, 3) extending the superstimulation protocol by 3 days will allow follicles to mature better and 4) markers of maturity, cellular health and survival will be turned off by FSH starvation.

The objective of the first study (Chapter 3) was to determine the effects of extending the length of superstimulation and follicular aging on oocyte competence by in vitro embryo production. Multiple follicles were allowed to grow for 4 (Short FSH) or 7 days (Long FSH) under the treatment of 8 or 14 injections of FSH (at 12-hour intervals), respectively. Multiple follicles in the FSH starvation group were allowed to grow for 7 days but FSH was provided for only the first 4 days of superstimulation. Extending the duration of follicular growth by superstimulation resulted in a greater number of ≥9 mm follicles and in 2.5 more transferable embryos per animal (morulae+blastocysts) at Day 9 of in vitro embryo culture. The FSH starvation resulted in a greater proportion of poor quality oocytes lower cleavage rate and lower embryonic development.

Microarray analysis was used to assess the effect of superstimulation (Chapter 4), follicular aging (Chapter 5) and FSH starvation (Chapter 6) on the gene expression profile of superstimulated granulosa cells. Gene expression of granulosa cells from the post-LH preovulatory dominant follicle was compared (Chapter 4) with those from follicles of the same status after a standard 4-day superstimulation (same protocol as Short FSH group from Chapter 3). A total of 190 genes were down-regulated and 280 genes were upregulated in the superstimulated group when compared with the reference

(non-superstimulated control). Data analysis showed that superstimulated follicles are still in a growing phase compared to untreated dominant follicles (most of the upregulated genes are related to matrix remodeling due to tissue proliferation) and did not respond to LH properly (down regulation of LH gene markers). Four-day superstimulation also disturbed genes related to angiogenesis and activated oxidative stress response genes. Extending the superstimulation protocol (7 days; same protocol as Long FSH from Chapter 3) allowed more time for follicles to leave the growing stage and properly respond to LH surge (most of the upregulated genes in the Long FSH group are markers of post LH surge) when compared to the standard 4 day superstimulation protocol (Short FSH; reference group) (Chapter 5). Moreover, the follicles from Long FSH show proximity to ovulation. The continuous FSH support during the extended superstimulation protocol is crucial for follicular health since FSH starvation disturbed genes markers of oocyte quality and embryo development (Chapter 6). Granulosa cells that underwent FSH starvation do not respond to LH surge, which could be detrimental to ovulation (Chapter 6).

Therefore, follicles from Short FSH are delayed in maturation and differentiation but the oocyte competence is not compromised. Extending superstimulation protocol by 3 d enhanced the ovarian response to FSH treatment and allowed more time for follicles to mature and properly respond to the LH stimulus. A period of FSH starvation after superstimulatory treatment compromised follicular health, ability to respond to LH and ovulate, oocyte quality and the fertilization process.

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Dedication

I would like to dedicate this thesis to the members of the family Faustino Prado (Thiago, Maria Luiza and Julie). I am proud to be part of this family. Love you all.

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LIST OF ABBREVIATIONS

CIDR	Controlled internal drug releasing device
COC	Cumulus oocyte complex
DNA	Deoxyribonucleic acid
dPBS	Dulbecco's phosphate-buffered saline
d	Days
E_2	Estradiol
FSH	Follicle stimulating hormone
h	Hours
IVF	*In vitro* fertilization
im	Intramuscular
LH	Luteinizing hormone
µg	Microgram
mg	Milligram
mm	Millimeter
ng	Nanogram
pg	Picogram
P_4	Progesterone
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PCR	Polymerase chain reaction
GV	Germinal vesicle
GVBD	Germinal vesicle breakdown
CL	Corpus luteum
RNA	Ribonucleic acid
RT-PCR	Real time- polymerase chain reaction
vs	Versus

CHAPTER 1

1. GENERAL INTRODUCTION

Understanding what affects oocyte competence is a major advantage when developing strategies to improve oocyte competence. In other words, what makes a good oocyte? The question seems simple but the answer is certainly not. Answering this question is the key to improving fertility in livestock animals either during controlled breeding or the natural cycle.

Cattle can have either 2 or 3 follicular waves during the estrous cycle (Adams 1999). Females with 2 waves have shorter interovulatory interval but longer duration of follicular waves (2-3 days longer) (Ginther *et al.* 1989c). Thus, the ovulatory follicles grow longer in 2-wave versus 3-wave cycles, and they have a larger diameter at ovulation (Ginther *et al.* 1989c). Moreover, prior to luteolysis, ovulatory follicles grow under a high-progesterone environment for 6 versus 3 days in 2- and 3-wave cycles, respectively (Adams 1999, Mihm *et al.* 2002). After luteolysis, the progesterone free period is similar in 2 and 3-wave cycles (3 days) (Adams 1999, Mihm *et al.* 2002). In summary, the ovulatory follicle from 2-wave cycles grows 3 days longer under a high progesterone environment and could be considered aged. The longer duration of the growing and static phase of the ovulatory follicle is termed follicular aging and may affect oocyte competence.

Superstimulation is a technique that has been widely used in research programs since it allows increasing experimental units by increasing ovarian outcome. With the indiscriminate use of superstimulation for research purposes the follicle that underwent superstimulation has been routinely used as a control. However, scientists do not fully know if superstimulated follicles or oocytes are truly comparable to a natural cycle. Thus, the effect of superstimulation treatment on the follicular environment and oocyte quality is not well understood. There is need to first determine if and to what extent superstimulation affects follicles and oocytes; and later to determine the best

superstimulation protocol. The best superstimulation protocol should be the one that most correlates with follicles and oocytes from the natural cycle and also results in an optimized response.

Current parameters used for determining oocyte competence are based on oocyte morphology, and the ability to fertilize and to develop to the blastocyst stage. However, newer technologies that assess the molecular status of follicles and oocytes allow a deeper approach to determine oocyte competence.

The following sections review the literature regarding bovine estrous cycle, control of reproductive axis, superstimulation and the molecular changes during follicle dynamics in cattle.

1.1 Overview of estrous cycle

The female reproductive life starts with the onset of puberty, which is the establishment of the first estrous cycle. The estrous cycle is defined by the ovarian and hormonal changes between two consecutive estrus events. It provides females with recurrent opportunities to copulate and become pregnant. The onset of puberty occurs on average at the age of 15 months in heifers; however it varies depending on factors such as breed or genotype, body weight and nutrition, social environment, and season (Youngquist & Threlfall 2007). The duration of the estrous cycle in cattle averages 21 days (Senger 2005, Youngquist & Threlfall 2007). The estrous cycle can be divided into four different phases: proestrus, estrus, metestrus and diestrus. The proestrus phase averages 2 days and is defined by the period that immediately precedes estrus. This phase is characterized by regression of the corpus luteum (CL) from the previous estrous cycle to the onset of estrus. Therefore it is a period of great hormonal transition, from progesterone to estrogen phase. Estrus is the period that the female is sexually receptive, or "in heat". It is the most recognizable phase as animals have a specific visible behaviour. Estrus behaviour can last up to 20 hours. Spontaneous ovulation occurs approximately 28 hours after the onset of estrus, and is followed by development of a CL. Metestrus is characterized by early development of the CL (average, 3 days), therefore by the hormonal transition from estrogen to progesterone. Diestrus is the period when the

CL is fully developed and active. The total length of diestrus is directly related to the time the CL remains functional. If animals do not become pregnant, the CL lasts up to 15 days. However, the CL can be maintained until parturition if the animal becomes pregnant.

1.2 Folliculogenesis

The process whereby immature follicles grow into large preovulatory follicles is termed folliculogenesis. There are five different stages of ovarian follicle development: primordial, primary, secondary, tertiary and preovulatory (Ireland 1987, van den Hurk & Zhao 2005). Primordial follicles are microscopic structures, which contain the most immature oocyte (van den Hurk & Zhao 2005). A flat single layer of squamous (pre-granulosa) cells surrounds the immature oocyte. The primary follicle is slightly more mature and can be identified by the change in the shape of granulosa cells, from flat to cuboidal (van den Hurk & Zhao 2005). As the follicles and oocytes continue to grow, the number of layers of granulosa cells surrounding the follicle increase. The main characteristics of the secondary follicles are the presence of 2 or more layers of granulosa cells and the formation of the theca layer and the zona pellucida (van den Hurk & Zhao 2005). The theca layer surrounds the outmost follicular layer and will be further divided into theca interna and externa (Ireland 1987). Networks of capillaries form between and within these two layers of theca cells; blood can now circulate from and to the follicle, directly. Secondary follicles will also have a capsule of glycoproteins, which is formed around the oocyte and is termed the zona pellucida (van den Hurk & Zhao 2005). Up to 6 layers of granulosa cells will surround this follicle (Ireland 1987). The most developed follicle is the tertiary. The main characteristic of the tertiary follicle is the formation of a fluid-filled antrum (Ireland 1987). Tertiary follicles can be classified as early or late tertiary, and is also known as a vesicular, antral, or Graafian follicle. Late tertiary follicles can reach ≥15 mm in diameter and may become the ovulatory follicle.

1.3 Follicular dynamics

With the advancement in technology, specifically the use of ultrasonography, changes in ovarian morphology could be sequentially monitored, with essentially no interference on function. This provided the confirmation that follicles develop in a wave-like pattern (Pierson & Ginther 1984, Pierson & Ginther 1988). The bovine estrous cycle is composed of either two or three follicular waves (Pierson & Ginther 1984, Savio *et al.* 1988). Didactically, the estrous cycle can be divided into two phases based on the most prominent structure present at the ovaries: the luteal and follicular phase (Senger 2005, Youngquist & Threlfall 2007). The luteal phase is when a CL is present and actively producing progesterone; it is a longer phase as it represents 80% of the estrous cycle (Senger 2005, Youngquist & Threlfall 2007). The follicular phase is after luteolysis when a dominant follicle is present and producing estradiol; it encompasses no more than 20% of the estrous cycle (Senger 2005, Youngquist & Threlfall 2007). Although there are these two-phase distinctions, the follicles grow and regress throughout the estrous cycle. The processes of follicular growth and regression (atresia) are termed follicular dynamics.

Follicular dynamics resumes by the recruitment of follicles (the follicular cohort); followed by the selection of a dominant follicle from the follicular cohort and atresia of subordinate follicles (Pierson & Ginther 1988, Sirois & Fortune 1988, Knopf *et al.* 1989). After selection the dominant follicle undergoes growing, static and regression stages or, if under a follicular phase, will become the pre-ovulatory follicle and ovulate (Pierson & Ginther 1988, Sirois & Fortune 1988, Knopf *et al.* 1989).

1.3.1 Wave theory

Cattle can have either 2 or 3 follicular waves during the estrous cycle (Pierson & Ginther 1984, Pierson & Ginther 1988, Ginther *et al.* 1989c, Knopf *et al.* 1989). The number of waves does not appear to be related to factors such as breed, age, or season; but it seems to be repeatable within individuals (Jaiswal *et al.* 2009). Females with 3 waves have a longer interovulatory interval (IOI) than females with 2 follicular waves (22-24 days versus 18-20 days, respectively) (Ginther *et al.* 1989c). In the first wave,

follicles emerge (Day 0), grow for 1-2 days, one follicle is selected to be the dominant follicle (at approximately Day 3), which grows until approximately Day 6, reaches a plateau (no change in diameter), and ultimately undergoes regression (starting at approximately Day 12) (Ginther *et al.* 1989c). A second wave emerges at Day 10 in 2-wave cycles, and at Days 8 or 9 in 3-wave cycles; it becomes the ovulatory wave in 2-wave cycles (Ginther *et al.* 1989c). In 3-wave cycles, a third wave emerges at approximately Day 16 or 17 and is the ovulatory wave (Ginther *et al.* 1989c).

Ovulatory follicles grow longer in 2-wave versus 3-wave cycles. The main difference in the duration of the growth of ovulatory follicles between 2 and 3-wave cycles is in the period prior to luteolysis, under a high-progesterone environment (6 versus 3 days in 2- and 3-wave cycles, respectively) (Ginther *et al.* 1989c, Mihm *et al.* 2002, Jaiswal 2007, Dias *et al.* 2012b). After luteolysis, ovulatory follicles grow for similar durations (3 days) in 2 and 3-wave cycles. Therefore ovulatory follicles from 2-wave cycles grow 3 days longer under a high progesterone environment, and they have a larger diameter at ovulation than those of 3-wave cycles (Ginther *et al.* 1989c, Mihm *et al.* 2002, Jaiswal 2007, Dias *et al.* 2012b).

1.4 Temporal relationship between follicle development and systemic endocrine system

Successful reproduction is highly dependent on signaling interactions within the hypothalamic-pituitary-gonadal axis. The hypothalamus produces and secretes gonadotrophin-releasing hormone (GnRH), a neurohormone that acts in the anterior pituitary to modulate the release of follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Baenziger & Green 1988, Senger 2005). The gonadotropins enter the peripheral circulation to stimulate the ovaries (Baenziger & Green 1988, Senger 2005). In the ovaries, the dominant follicle produces estradiol, whereas the corpus luteum (CL) produces progesterone (Senger 2005). The effect of progesterone and estradiol on the pattern of GnRH secretion determines the stimulation or suppression of FSH/LH released from the pituitary (Glencross 1987). This effect of steroid hormones on the GnRH-

induced gonadotropin is termed feedback, and it varies in a positive or negative manner throughout the estrous cycle.

For each wave, a group of follicles emerges after an increase in peripheral FSH concentrations (Adams *et al.* 1992b). The estradiol produced by the growing cohort, particularly the dominant follicle, signals through GnRH neurons in the hypothalamus and at the level of the pituitary gland and to suppress FSH concentrations (Gibbons *et al.* 1997). When circulating FSH begins to decline, subordinate follicles stop growing and become atretic (Gibbons *et al.* 1997, Ginther *et al.* 1999). However, the dominant follicle acquires LH receptors in its granulosa cells and is, therefore, no longer dependent on FSH (Bao *et al.* 1997, Ginther *et al.* 1999, Luo *et al.* 2011). In the first follicular wave a CL from a previous ovulation is active. The high levels of progesterone exert a negative feedback on GnRH-induced LH secretion, thus preventing the pre-ovulatory LH surge (Ginther *et al.* 1989b, Rathbone *et al.* 2001, Mihm *et al.* 2002). The dominant follicle of wave 1 consequently regresses and a new wave emerges (Adams *et al.* 1992a, Mihm *et al.* 2002). However, after luteolysis, progesterone levels are low and estradiol from the pre-ovulatory dominant follicle is high. The high estradiol levels reach peripheral circulation and signal through GnRH neurons, increasing LH pulse frequencies to obtain a pre-ovulatory LH surge (Ginther *et al.* 1998, Rathbone *et al.* 2001).

1.4.1 Hypothalamic hormone

The hypothalamus produces and secretes the GnRH, a neurohormone that acts on the anterior pituitary to modulate the release of FSH and LH. Two distinct areas in the hypothalamus control the secretion of GnRH: the tonic and surge center (Senger 2005). The tonic center is located at the arcuate nuclei in the hypothalamus and is responsible for basal secretion of GnRH (Kallo *et al.* 2012). The neurons in this area release GnRH in small pulses throughout a sustained period of time. In contrast, the surge center is located at the preoptic nuclei in the hypothalamus (Kallo *et al.* 2012) and produce GnRH in high frequency pulses for a short period of time. The surge center is present only in females and is responsible for the pre-ovulatory LH surge (Senger 2005).

Many factors determine whether GnRH will affect the secretion of LH or FSH from the pituitary; however the stage of the estrous cycle is the main determinant (Adams *et al.* 2008). In different words, the type of steroid signaling influences the pattern of GnRH pulses (Kinder *et al.* 1991, Adams *et al.* 2008), which determines LH or FSH secretion. The density of GnRH receptors in the anterior pituitary also reflect LH or FSH stimulus (Senger 2005). A high density of GnRH receptors is associated with LH secretion, while a low density is associated with FSH secretion. Understanding of the interaction among hormones from the hypothalamus-pituitary-gonadal axis is crucial for animal reproduction studies.

1.4.2 Pituitary-gonadotropins hormones

LH and FSH are both produced and released by the gonadotropes in the anterior pituitary gland and are termed gonadotropins (Baenziger & Green 1988). In the anterior pituitary the gonadotropic cells are located in the pars distalis and sparsely at the pars tuberalis. They are both glycoproteins that contain 2 polypeptide subunits, alpha (α) and beta (β) (Baenziger & Green 1988). The α subunit is common for both hormones. However, the β subunit is hormone-specific (Baenziger & Green 1988).

LH is mainly responsible for causing ovulation, luteinizing follicular cells and stimulating luteal cells to produce progesterone; while FSH is mainly responsible for follicular growth (Ginther *et al.* 1998). LH and FSH are also crucial during follicle selection (Ginther *et al.* 1996, Ginther *et al.* 1998, Ginther *et al.* 2001). The follicular cohort is dependent on FSH. Differential growth of a dominant follicle normally occurs when the largest follicle reaches 8.5 mm in diameter (Ginther *et al.* 1997). The future dominant follicle is the first to acquire LH receptors in the granulosa cells (Luo *et al.* 2011); it starts to produce higher levels of estradiol and inhibin, which suppress FSH (Adams *et al.* 1992a, Adams *et al.* 1993a). When FSH reaches a nadir, subordinate follicles stop growing and become atretic. Receptors for LH can be first detected in granulosa cells from 2 to 4 days after wave emergence, which is the expected time of follicular selection (Bao & Garverick 1998, Luo *et al.* 2011). In addition, an increased concentration of LH at the time of deviation was reported in heifers (Bao *et al.* 1997).

If high levels of FSH are maintained, selection does not occur. In this case, many follicles from the follicular cohort are able to grow to a pre-ovulatory stage (Mapletoft *et al.* 2002).

The LH surge is also associated with the final growth and maturation of the dominant follicle (Eppig 1996). The resumption of meiosis is the main event following an LH surge and is termed oocyte nuclear maturation (Eppig 1996).

1.4.3 Gonadal hormones

Estradiol is a gonadal hormone produced by the follicles and like any other steroid hormone is derived from cholesterol. It is produced by the "two-cell two-gonadotropin" system by the granulosa and theca cells of the follicular wall under the influence of LH and FSH (Senger 2005, Youngquist & Threlfall 2007). The LH binds to its receptor on theca cells, which activates a cascade of events that results in the conversion of cholesterol into testosterone (Senger 2005, Youngquist & Threlfall 2007). Testosterone crosses the basal membrane and enters the granulosa cells. When FSH binds to its receptor on granulosa cells it activates a cascade of events that culminate in conversion of testosterone to estradiol (Senger 2005, Youngquist & Threlfall 2007).

The effect of estradiol on the pattern of GnRH secretion determines the stimulation or suppression of FSH/LH released from the pituitary (Adams *et al.* 2008). Estradiol has a negative effect on the basal secretion of LH and FSH by acting through the tonic center of the hypothalamus (Adams *et al.* 2008). However, the surge center of the hypothalamus responds dramatically to estradiol with the pre-ovulatory LH surge when under a low progesterone environment (Adams *et al.* 2008).

Estradiol has been suggested to play an important role at the time of deviation (Ginther *et al.* 1998, Kulick *et al.* 1999, Ginther *et al.* 2000). The increased level of estradiol secretion by the future dominant follicle at the time of deviation is associated with acquisition of LH receptors in the granulosa cells and suppression of FSH (Adams *et al.* 1993b). Thus, at the time of deviation the future dominant follicle transitions the dependency from FSH support to LH. The ablation of the dominant follicle removes the

negative effect of estradiol on FSH release and induces a new wave emergence 1.5 d later (Bergfelt DR *et al.* 1994).

The highest level of estradiol occurs at estrus. At this stage estradiol is responsible for the estrus behaviour, which is increased locomotion, phonation, nervousness, attempts to mount other animals, lordosis and sexual receptivity (Morrow 1969, Reames *et al.* 2011).

During ovulation the basement membrane between granulosa and theca cells disintegrates and the follicular wall collapses into folds (Senger 2005). Thus, the physical separation of granulosa and theca cells disappears. Under the effect of the pre-ovulatory LH surge granulosa and theca cells luteinize. Three to five days after ovulation, the newly formed luteal tissue starts to produce progesterone (Mihm *et al.* 2002). Progesterone is a steroid hormone derived from cholesterol and is responsible for maintenance of pregnancy (Senger 2005). Levels of progesterone are elevated until late diestrus, when luteolysis occurs and progesterone levels drop. Luteolysis occurs as a consequence of prostaglandin ($PGF_{2\alpha}$) released from the endometrium (Senger 2005). A clear understanding of the effects of P_4 and $PGF_{2\alpha}$ on the estrous cycle is crucial for estrous synchronization.

Progesterone has negative effects on the hypothalamus, suppressing GnRH at the tonic and surge center (Mihm *et al.* 2002). Although progesterone has a negative effect on both LH and FSH, increases of FSH concentration naturally occur under a high progesterone environment (Mihm *et al.* 2002). The same does not happen with LH secretion; high progesterone inhibits the LH surge (Mihm *et al.* 2002). Thus, progesterone levels determine if a follicle will ovulate or regress.

1.5 Oocyte maturation versus oocyte competence

Oocyte competence is the ability of an oocyte to successfully develop, be fertilized and develop into a blastocyst. Oocyte maturation is a crucial part of oocyte development, without it cannot gain competence. It is usually segregated into nuclear and cytoplasmic events.

1.5.1 Nuclear maturation

Nuclear maturation is defined as the ability of the oocyte to resume meiosis. Primary oocytes within the primordial follicles undergo meiosis while still in the fetal ovary (Fuhrer *et al.* 1989), however, meiosis is arrested at prophase I (first meiotic arrest) (Fuhrer *et al.* 1989). After primordial follicle activation, oocytes develop and increase in volume. The ability to resume meiosis is gained when oocytes reach a critical minimum size and follicles form an antrum (tertiary follicles) (Fair *et al.* 1995). However, meiosis resumes only after a pre-ovulatory LH surge. Shortly after the LH surge, the gap junctions between cumulus cells and the oocyte deteriorate (Hyttel *et al.* 1986, Tornell *et al.* 1990). Cumulus cells are thought to produce meiosis-inhibitory factors that cross those gap junctions and target the oocyte (Hyttel *et al.* 1986, Tornell *et al.* 1990). The loss in communication between cells removes the inhibitory signalling and the oocyte can than resume meiosis. However, meiosis is again stopped at metaphase II (second meiosis arrest) until fertilization, when meiosis is finally completed.

The resumption of meiosis is a complex process that can be described and classified in different ways. The classification based on morphology of chromosome appearance is commonly used. Before maturation, oocytes contain a large germinal vesicle (nucleus) with a large nucleolus, which suggests that the cell is transcriptionally active (Chohan & Hunter 2003, Liu *et al.* 2006). At this stage (GV), the chromosomes are decondensed and disperse (Chohan & Hunter 2003, Liu *et al.* 2006). After the LH surge, maturation progresses further with chromosomes starting to condense, germinal vesicle breakdown and the nucleolus disperses; this stage is termed germinal vesicle breakdown (GVBD) (Hyttel *et al.* 1986).

1.5.2 Cytoplasmic maturation

Oocyte cytoplasmic maturation involves a cascade of events that will give the oocyte the ability to complete nuclear maturation, fertilization and early embryonic development. Although few studies (Assey *et al.* 1994, Ferreira *et al.* 2009) are now focusing on better understanding the mechanisms regulating cytoplasmic maturation, it is

still a poorly defined topic. It refers to the changes in organelle morphology and functions to optimize storage of mRNA, proteins, substrates, and nutrients (Ferreira *et al.* 2009).

1.6 Persistent and over-dominant follicles

The gross definition of a persistent follicle is a follicular-like structure that is larger than 25mm and persists in the ovary for over 10 days (Kesler & Garverick 1982). The effect of steroid feedback on the pattern of LH pulsatility plays a major role in the formation of persistent follicles (Kesler & Garverick 1982). Normally, the high levels of estradiol and low levels of progesterone during the follicular phase increase the LH pulse frequency and ultimately lead to an LH surge and ovulation (Adams *et al.* 2008). However, the pathophysiology of persistent follicles consists of increased LH pulse frequency during sub-luteal levels of progesterone and high levels of estradiol (Kesler & Garverick 1982). The sub-luteal levels of progesterone prevent ovulatory LH surge and without ovulation the follicle persist (Revah & Butler 1996). Increased incidence of persistent follicles occurs with the use of a prolonged treatment of progesterone during estrous synchronization programs. Feeding animals with melengestrol acetate (MGA) for 14 days results in large persistent follicles and consequently decreased pregnancy rates due to ovulation and fertilization failure (Zimbelman & Smith 1966, Custer *et al.* 1994, Rathbone *et al.* 2001). Thus, prolonged treatment with MGA with the intent to synchronize estrus was discouraged due to a great economical loss (Revah & Butler 1996).

Similarly, ovulatory follicles from animals that have 2 follicular waves during the estrous cycle also grow longer and to a larger size than follicles from animals that have 3 follicular waves (Adams *et al.* 2008, Dias *et al.* 2012b). Those over-dominant follicles may be considered aged, which could compromise oocyte competence. Plasma levels of progesterone and estradiol do not differ during the growth period of ovulatory follicles of 2 or 3-wave cycles (Noseir 2003, Dias *et al.* 2012b). The major difference is in relation to the time of luteal regression, with cows from 2-wave cycles maintaining the ovulatory follicle for a longer period under a high progesterone environment (Ahmad *et al.* 1997). It was long believed that animals with 2-wave cycles had decreased fertility (Ahmad *et al.*

1997, Townson *et al.* 2002, Bleach *et al.* 2004). However, this long-standing notion was disproved by a large report that failed in detecting an effect of the number of waves on fertility in cattle (Dias *et al.* 2012b).

Therefore, the major difference between persistent follicles and over-dominant follicles is regarding progesterone and the effect on fertility. Extended luteal phase accounts for over-dominant follicles (as in 2-wave cycles) (Ahmad *et al.* 1997), while prolonged sub-luteal levels of progesterone accounts for persistent follicles (Kesler & Garverick 1982). Persistent follicles are associated with low fertility (Revah & Butler 1996), while over-dominant follicles (as in 2-wave) are not (Dias *et al.* 2012b).

1.7 Superstimulation and superovulation

Maintenance of high concentrations of FSH prevents dominant follicle selection. Thus, the superstimulation principle is based on prolonging the effect of FSH, which may prevent atresia of many follicles and ultimately allow many to ovulate (Mapletoft *et al.* 2002). Therefore, superstimulation rescues follicles that would otherwise undergo atresia with the final intent to maximize fertilized ova and transferable embryos (Mapletoft *et al.* 2002).

The main limitation is the individual variability in the superstimulatory response (Looney 1986, Adams *et al.* 1994), which has a great negative impact on the cost of embryo transfer programs. However, other limitations such as time and effort required for superstimulation treatment and estrus detection are also encountered (Mapletoft *et al.* 2002). Variation of superstimulatory response has low heritability (Tonhati *et al.* 1999), which compromises selection for genetic improvement programs. High individual variation can be attributed to environmental factors related to reproductive, sanitary or nutritional conditions, and the type and duration of gonadotropin used (Mapletoft *et al.* 2002). However, the specific factors that contribute to the high individual variation are not fully known.

Many approaches to minimizing the variability in superstimulatory response have been tested (Nasser *et al.* 1993, Adams 1994, Adams *et al.* 1994). The major determinants of the superstimulatory response are the number of follicles available at

wave emergence (Singh *et al.* 2004) and the timing of the onset of treatments in relation to wave emergence (Nasser *et al.* 1993). A greater ovulatory response is achieved when superstimulation treatment is started at the time of wave emergence (Nasser *et al.* 1993).

Superstimulation has been widely used in animal breeding programs (Mapletoft *et al.* 2002), but the effect of superstimulation treatment on the follicular environment and oocyte quality is also not well understood. A common use of superstimulation is to increase experimental units (oocyte, embryos or follicular cells) for research purposes. However, if the status of follicles and oocytes that underwent superstimulation are comparable to those from the natural cycle is also not known.

1.7.1 Protocols for superstimulation

Equine chorionic gonadotrophin (eCG) was first used to obtain an ovarian superstimulated response in cattle (Mapletoft & Bo 2011). However, the number and quality of embryos obtained after protocols involving eCG was low (Monniaux *et al.* 1983). The recommended dose of eCG is 2500 IU, but this results in active concentration in plasma for up to 10 days and causes excessive superstimulation (Mapletoft *et al.* 2002). The eCG has a primary FSH activity with varying degrees of LH activity. Researchers associated the poor response to eCG superstimulation protocols with the high LH content (Moor *et al.* 1984, Mapletoft *et al.* 2002). To maximize the superstimulation ovarian response the maximum LH contamination is 20% (Mapletoft *et al.* 2002).

Another approach is using purified porcine pituitary extract (pFSH). This preparation was considered free of LH contamination, therefore improved results can be obtained (Chupin *et al.* 1984, Looney *et al.* 1988, Willmott *et al.* 1990). However, one disadvantage of protocols involving pFSH is the short half-life of this drug (5 hours) (Laster 1972). For that reason, multiple injections need to be given to induce an optimal stimulatory response.

A popular superstimulation protocol involves three main steps: 1) synchronization of a new follicular wave, 2) initiation of the superstimulation protocol at the same day of wave emergence and 3) synchronization of superovulation (Mapletoft *et al.* 2002,

Mapletoft & Bo 2011). To synchronize a new wave is the key for successful superstimulation response as the highest response to superstimulatory treatment in cattle is obtained when treatment is initiated at the time of wave emergence (Nasser *et al.* 1993, Adams 1998). Treatments with progesterone plus estradiol or the physical ablation of a dominant follicle are both commonly used methods to synchronize a new follicular wave, along with the insertion of a CIDR-B (Jaiswal 2007). Depending on the methods used for wave synchronization the initiation of pFSH treatment can be 4 or 1 day later (for progesterone plus estradiol or follicular ablation method, respectively) (Bergfelt DR *et al.* 1994, Martinez *et al.* 2000). The pFSH treatment is given twice daily of constant or decreasing dose, for 4 to 5 days, with $PGF_{2\alpha}$ injection and CIDR removal on the last day of FSH treatment (Mapletoft *et al.* 2002, Baruselli *et al.* 2006). A good superovulation synchrony is crucial for embryo transfer programs. In that regard, to induce superovulation exogenous LH is given 24 hours after CIDR removal (Baruselli *et al.* 2006).

The need for twice-daily treatment of pFSH can be time-consuming, stressful and lead to error, especially when working with large group of animals. One study (Tribulo *et al.* 2012) reported that the use of a traditional twice-daily pFSH protocol or a single intramuscular dose of pFSH combined with hyaluronan-based slow-release formulation (SRF) results in similar superstimulation responses.

A superstimulation model has also been used to examine the effects of progesterone and the duration of the follicular growing phase on oocyte competence (Dias *et al.* 2012a). Studies report that extending the period of progesterone exposure during the growing and early-static phase of the ovulatory follicle by 3 days did not affect oocyte competence of a single dominant follicle (Dias *et al.* 2012b) or when under superstimulation (Dias *et al.* 2012a). However, 3 days longer progesterone exposure during superstimulation resulted in better ovarian response (Dias *et al.* 2012a, Garcia Guerra *et al.* 2012) to the treatment.

1.7.2 FSH starvation

FSH starvation refers to the interval from the end of FSH treatment to ovulation or collection of oocytes; it is also referred to as FSH withdrawal or coasting period (Blondin *et al.* 1997b, Sirard *et al.* 1999, Dias *et al.* 2012a, Garcia Guerra *et al.* 2012).

Some have suggested a beneficial effect of FSH starvation ("coasting") on oocyte competence after superstimulation (Blondin *et al.* 1997b, Sirard *et al.* 1999). A greater proportion of oocytes developed into embryos *in vitro* after a 48-hour period of FSH starvation compared to 24 and 72 h (Blondin *et al.* 1997b). However, a similar beneficial effect could not be replicated using 48 h of FSH starvation in *Bos indicus* animals (Nogueira *et al.* 2007). Longer periods of FSH starvation resulted in a loss of ovulatory capability (Jaiswal R.S. *et al.* 2006, Dias *et al.* 2012a). In one study, 144 hours of FSH starvation resulted in loss of ovulatory capability in 100% of follicles that developed in a low progesterone environment (Jaiswal R.S. *et al.* 2006). Another study reported 50% of loss in ovulatory capability from follicles that developed under luteal-phase levels of progesterone and after 96 hours of FSH starvation (Dias *et al.* 2012a). In those studies oocyte competence could not be assessed due to the lack of ovulation. In a recent study (Nivet *et al.* 2012), 92 h of FSH starvation resulted in an increase in the number of ≥10 mm follicles, but blastocyst formation rate was reduced compared to 44 and 68 h. These studies indicate that oocytes undergo certain undefined changes during FSH starvation and depending on the duration of FSH starvation those changes could increase oocyte competence or lead to loss of ovulatory capability.

Perhaps a good research approach is to compare the effect of FSH starvation with the effect of continuous FSH support on oocyte competence but using the same duration of superstimulation protocol. Moreover, the detailed molecular mechanism involved in FSH starvation is not yet described.

1.8 Overview of molecular changes in ovarian cells during follicular dynamics

Cattle are a monovulatory species, which means that normally only a single dominant follicle ovulates per estrous cycle (Youngquist & Threlfall 2007). Therefore,

selection of a single dominant follicle from the follicular cohort is part of the normal follicular dynamics, while subordinate follicles (follicles that do not get selected) undergo regression (Youngquist & Threlfall 2007). The selected dominant follicle undergoes growing, static and regression stage or if under a follicular phase will become the pre-ovulatory follicle and ovulate (Youngquist & Threlfall 2007). Each of these stages are preceded or accompanied by changes in gene expression of follicular cells and oocytes. Understanding the complex changes in gene expression while follicles develop is of great importance to better understand follicular dynamics.

1.8.1 Genes relates to selection

Follicular deviation in cattle occurs at the time that the largest follicle in the ovary reaches on average 8.5 mm diameter, approximately at day 3 of estrous cycle (Ginther *et al.* 1996). Luteinizing hormone seems to be crucial during follicular selection. Around the time of follicle deviation the expression of mRNA for LH receptor (LHr) increases up to 7-fold in the granulosa cells of the future dominant follicle (Mihm *et al.* 2006, Nogueira *et al.* 2007, Luo *et al.* 2011). This is considered the earliest and most dramatic change between the expected future dominant follicle and the expected future subordinate follicle (Luo *et al.* 2011). Moreover, all dominant follicles express mRNA for LHr. Thus, it is strongly suggested that the acquisition of LHr in granulosa cells is required during follicle selection and dominance. LHr is also present in theca cells and when stimulated by the binding of LH induces the expression of steroidogenic genes, such as STAR, CYP11A1 and CYP17A1 (Luo *et al.* 2011). Those steroidogenic enzymes produce androgen substrates for aromatase. Another early event during follicle selection is the increase in expression of CYP19A1 in granulosa cells and consequently the increase in E2 concentrations in follicular fluid (Luo *et al.* 2011).

The use of a GnRH antagonist (acyline) before follicle selection inhibits LH pulses, which completely suppresses the induction of LHr expression in granulosa cells (Luo *et al.* 2011). This suggests that LH may promote the expression of its own receptor. Without the LH stimulus the expression of steroidogenic genes such as STAR and

CYP17A1 in theca cells was down-regulated; therefore the estradiol concentration in follicular fluid was also decreased (Luo *et al.* 2011).

The insulin growth factor family is also critical for follicle growth and selection (Fortune *et al.* 2004, Ginther *et al.* 2004, Mihm & Evans 2008). Free IGF1 is higher in the future dominant follicle than the future subordinate follicle even before selection has occurred (Ginther *et al.* 2001). The increase of IGF1 in follicular fluid coincides with the increase of estradiol concentrations and with the decrease of IGFBP (Insulin growth factor binding proteins) (Beg *et al.* 2001, Ginther *et al.* 2001, Rivera & Fortune 2003). IGF binding proteins binds to the IGF, decreasing the availability of free IGF. The decrease in IGFBP activity is controlled by a protease PAPP-A (pregnancy related plasma protein A), which is also high in follicular fluid at the time of deviation (Liu *et al.* 2009, Luo *et al.* 2011). IGF1 knockout mice had follicles arrested in pre-antral stage with CYP19A1 expression reduced (Zhou *et al.* 1997). Studies involving tissue culture confirmed the effect of IGF on enhancing steroidogenesis (reviewed in (Glister *et al.* 2001)). Moreover, in vivo intra-follicular injection of IGF1 resulted in increased estradiol concentration in follicular fluid to levels similar to the dominant follicle (Ginther *et al.* 2004). The expression of PAPP-A is decreased when a GnRH antagonist is used before follicular selection (Luo *et al.* 2011), suggesting that LH is associated with the control of PAPP-A expression, which is clearly involved in follicle selection.

Each follicular wave emergence is preceded by a rise in FSH levels (Adams *et al.* 1992b). When FSH starts to decline follicles are still growing. However, follicle selection occurs around 48 hours after an FSH peak (Mihm & Austin 2002). The decline in FSH prior to selection is caused by the production of inhibins by the FSH-dependent follicle cohort. The future dominant follicle increases the production of estradiol and inhibin A and then assumes the negative feedback on FSH release (Adams *et al.* 1993b, Adams *et al.* 1993a). Granulosa cells of the future dominant follicle also produce activins. Contrary to the inhibins, activins have an autocrine action promoting cellular growth and mediating the reduced requirement for FSH (Sisco & Pfeffer 2007). The gene Inh-βA encodes inhibin-βA peptide, which can homodimerise or heterodimerise to form activin A, activin AB or inhibin A (Sisco *et al.* 2003). One study showed a positive correlation of high expression of Inh-βA with future follicular dominance (Sisco & Pfeffer 2007).

Moreover, expression of Smad2 (a nuclear effector and putative target of the activin family) was also associated with dominant follicles (Sisco & Pfeffer 2007), highlighting the role of activin in follicular selection. However, in that study the levels of activin in follicular fluid did not correlate with dominance (Sisco & Pfeffer 2007), which clearly show the need for further investigation.

The involvement of known follicular genes such as the LH and FSH receptor, steroidogenic enzymes, specific growth factors and their binding proteins, on follicle selection is well established. However, global gene expression profiling was also used to determine key pathways that regulate follicular selection (Mihm *et al.* 2008, Liu *et al.* 2009). The functions and pathways associated with follicular selection are: cell proliferation and survival, lipid metabolism, tissue development, cell signaling and immune response (Mihm *et al.* 2008, Liu *et al.* 2009). Cyclin D2 (CCND2) is an important gene in cell proliferation pathway and is upregulated in future dominant follicles (Mihm *et al.* 2008). The protein encoded by CCND2 mediates the transition of G1 to S during mitosis. The expression of CCND2 is increased by E and IGF (Mihm *et al.* 2008), which are both high during follicular selection. Many genes that encode anti-apoptotic proteins such as GADD45, XIAP and FLIP are also upregulated in future dominant follicles compared to the future subordinate follicles (Mihm *et al.* 2008, Liu *et al.* 2009). However, the results from global gene expression profiling studies only suggest many hypotheses, opening doors for hypothesis-driven research type of projects. Therefore, further studies are needed for a better understanding of molecular mechanisms behind follicle selection.

1.8.2 Genes related to follicular regression

Follicular regression is the fate of all follicles from the follicular waves during the estrous cycle, except those that ovulate. Women have approximately 400 follicles attaining ovulation during the entire reproductive life, where 250,000 follicles undergo regression (a rate of almost 1000 atretic follicles per month) (Townson & Combelles 2012). Although those numbers are difficult to determine in cattle, the idea can be extrapolated.

The term follicular regression is mainly used to describe a process involving antral follicles within a wave (Townson & Combelles 2012). Thus, follicular regression can refer either to the subordinate follicles or to the non-ovulatory dominant follicle. This term is related but many times confounded with follicular atresia. Follicular atresia refers to the morphological changes leading to cell death in all follicles, including before the time of follicle formation or during follicular establishment (Forabosco *et al.* 1991).

Apoptosis is initiated either by the binding of death molecules to the cell surface death receptors or by intracellular signaling arising from mitochondria (Kerr *et al.* 1972, Hussein 2005). In the first case, death proteins such as tumor necrosis factor (TNF), Fas ligand, interferon gamma (IFN) and TNF-related apoptosis inducing ligand (TRAIL), bind to their respective receptor and initiate an apoptosis signaling cascade in the cytoplasm (Hussein 2005). Activation of three known cellular death domains is expected: the death domain (DD), the death effector domain and the caspase recruitment domain (CARD) (Hussein 2005). FLICE inhibitory protein (Flip) regulates the caspase pathway activation (Hussein *et al.* 2003a). This protein prevents apoptosis by inhibiting expression of caspase family proteins. Pro-apoptotic proteins such as the B cell/lymphoma-2 (Bcl-2), Bcl-2-associated protein X (BAX) and BH3 only domain proteins (Bad, Bid, Noxa and p53 upregulated modulator of apoptosis (PUMA)) facilitate the release of cytochrome *c* by the mitochondria (Hussein *et al.* 2003a, Hussein *et al.* 2003b, Hussein 2005). When cytochrome c is released into the cytoplasm it binds to one of the initiator caspases (caspase 8 or 9), which activate the caspase cascade (Hussein 2005). The caspase-family proteins cleave other proteins essential to cell viability and induce many changes associated with apoptosis (Hussein 2005).

Although many pro-apoptotic and anti-apoptotic proteins have been identified, the exact signal, interaction among those molecules and especially the time of apoptosis activation in relation to the follicular wave is still unclear. Briefly, it is known that: 1) multiples molecules are involved in apoptosis (Townson & Combelles 2012), 2) those molecules are divided into two categories, ones involved in survival of the cell (anti-apoptotic including gonadotropins, IGF1, fibroblast growth factor, TGF-α, etc.) and ones involved in triggering apoptosis (pro-apoptotic including TGF-β, interleukin-6, Bax, Fas, p53, TNF, caspases, etc.) (Townson & Combelles 2012), and 3) cellular fate depends

upon a shift in the balance of expression between pro and anti-apoptotic factors (Townson & Combelles 2012).

1.8.3 Matrix remodeling

The extracellular matrix is a complex structural matrix surrounding and supporting cells within a tissue; is also known as connective tissue (Alberts *et al.* 2002). It is composed mainly of structural proteins (elastin and collagens), specialized proteins (fibrillin, fibronectin, laminin) and proteoglycans (Alberts *et al.* 2002). It plays a prominent role in ovarian function by participating in processes such as cell migration, proliferation, growth, and development of follicles through an extensive chemical cellular signaling mechanism (Alberts *et al.* 2002). Matrix remodeling is also crucial during ovulation and CL formation.

The degree of matrix remodeling depends upon a disbalance between the expression of matrix metalloproteinase proteins (MMP) versus tissue inhibitor metalloproteinase proteins (TIMP) (Alberts *et al.* 2002, Murdoch & Gottsch 2003, Berkholtz *et al.* 2006b). MMPs are zinc-dependent endoproteinases that can degrade all kinds of matrix proteins. The MMPs form a large family with 28 different protein members (MMP1-28). They are grouped in four categories based on their targets and partially by the cellular location (Alberts *et al.* 2002): 1) collagenase (capable of degrading collagens), 2) gelatinase (capable of degrading collagen 4 and gelatin), 3) stromelysins (broad ability to cleave but unable to degrade fibrillar collagen) and 4) the membrane type MMP (characterized based on cellular location). TIMP is a natural inhibitor of the MMPs and is also known as collagenase inhibitor (Li & Curry 2009). There are four members in the TIMP gene family (TIMP1, 2, 3 and 4) and they all seem to inhibit MMPs (Imai *et al.* 2003, Li & Curry 2009). Conversely, TIMP proteins are known to induce expression of growth factors, promoting cellular growth and proliferation (Li & Curry 2009). The changes in gene expression of MMPs and TIMPs throughout the bovine estrous cycle have not been yet reported. However, MMP is expected to be upregulated during times that matrix remodeling is needed, for example during follicular growth, ovulation and CL formation (Imai *et al.* 2003).

Other genes such as THBS1, FN1, ADAMTS1, CTGF, HPSE, EGR1, PLAT, genes from the IGF family, and those involved in collagen formation are also involved in remodeling of the extracellular matrix. Some of those genes are mainly involved in matrix remodeling due to follicle growth (CTGF, SERPINE, FN1) (Colman-Lerner *et al.* 1999, Wandji *et al.* 2000, Harlow *et al.* 2002, Yasuda *et al.* 2005), while others are upregulated only at the time of ovulation (ADAMTS1, HPSE, EGR1, PLAT) (Galway *et al.* 1990, Leonardsson *et al.* 1995, Madan & Bridges 2003, Sayasith *et al.* 2006, Klipper *et al.* 2009).

Expression of the connective tissue growth factor (CTGF) mRNA is inversely related to follicular maturation in rats (Harlow *et al.* 2002) and pigs (Wandji *et al.* 2000). Thus, this gene is involved in matrix remodelling but perhaps only during follicular growth. Similarly, Fibronectin (FN1) expression is inversely linked to follicle development (Colman-Lerner *et al.* 1999, Yasuda *et al.* 2005, Berkholtz *et al.* 2006a), while SERPINE2 expression is found to be elevated pre LH surge in bovine granulosa cells (Gilbert *et al.* 2012)

Heparanase (encoded by HPSE) cleaves heparan sulfate glycosaminoglycans during matrix remodeling. HPSE is found to be highly expressed 12 hours post GnRH injection in bovine granulosa cells and it is suggested to be a novel member of the LH-induced ECM-degrading enzyme family, which will contribute to follicle rupture during ovulation (Klipper *et al.* 2009). Likewise, EGR1, PLAT and ADAMTS1 are also genes that were previously reported to be upregulated close or during ovulation. Plasminogen activator (PLAT) is a serine protease that converts plasminogen to plasmin, which is crucial during fibrinolysis (Sayasith *et al.* 2006). PLAT is highly expressed in granulosa cells of pre-ovulatory follicles in rats (Galway *et al.* 1990, Leonardsson *et al.* 1995). Expression of early growth response 1 (EGR1) activates a cascade of genes in the prostaglandin pathway and increased expression of LH receptor mRNA (Sayasith *et al.* 2006). However, EGR1 expression is maintained high for a short period; it increased after LH but is expected to decrease near the time of ovulation (Sayasith *et al.* 2006). ADAMTS1 is also known to cleave extra cellular matrix and is crucial in follicle rupture during ovulation and is upregulated after the LH surge (Madan & Bridges 2003, Peluffo *et al.* 2011). Markers of LH surge are described in the section below.

1.8.4 Markers of LH surge

The high levels of estradiol produced by the preovulatory follicle upregulate the expression of LH receptors and induce a LH surge through a positive feedback in the hypothalamus and pituitary (when under a low progesterone) (Adams *et al.* 2008). The LH surge induces in follicular cells a rapid, transient expression of genes essential to ovulation and corpus luteum formation (Richards 1994). Those genes that are specifically changed by LH surge are termed markers of LH surge.

1.8.4.1 Rapid response to LH surge

The immediate response after the binding of LH to the G protein receptor during a surge is the activation of adenylyl cyclase (AC) and thereby the increase in cyclic AMP (cAMP). Increased levels of cAMP will lead to stimulation of A- and C- kinase pathways (PKA or PKC). PKA and PKC will cause phosphorylation and activation of transcription factors such as the early growth regulatory factor 1 (Egr-1), the CAAT enhancer binding protein beta (C/EBPβ) and the cAMP response element-binding protein (CREB) (for a review see (Russell & Robker 2007)). Those factors are rapidly activated but they are transiently expressed (Russell & Robker 2007). The gene and protein expression of those transcription factors will peak at 4hrs post LH. The expression of other transcription factors such as the activator protein 1 family (Fos, Jun, Fra2, JunD) is also rapidly increased by the LH surge; however the high expression is sustained for a longer period (until ovulation) (Sharma & Richards 2000). The role of some of the transcription factors on ovulation are well documented, while others are not completely clear. For example, C/EBPβ control expression of COX-2 (Sirois & Richards 1993), which is a key gene oi the prostaglandin pathway. It was demonstrated that mice null for C/EBPβ do not ovulate (Sterneck *et al.* 1997). On the other hand, the specific role of Egr-1 on ovulation is not demonstrated yet.

The LH surge modifies the transcription machinery, which mediates the expression of peri-ovulatory genes. As a consequence of the LH surge granulosa cells rapidly produce Egf-L substrates as epiregulin, amphiregulin and betarcellulin. Those

factors are elevated 1-3 hrs after LH surge and are involved in transmitting the ovulatory signal to cumulus cells (Park *et al.* 2004). LH surge also indirectly induces the activation of progesterone and prostaglandin pathways (Murdoch & Gottsch 2003). Genes related to progesterone and prostaglandin pathways are induced by the LH surge and they have been implicated in ovulation (Richards *et al.* 1998). Deletion of progesterone receptor (PR) or prostaglandin endoperoxide synthase-2 (PTGS2) in genetically mutated mice results in an anovulatory phenotype (Lydon *et al.* 1995, Morham *et al.* 1995). Interestingly, the progesterone receptor is only expressed in pre-ovulatory follicles after the LH surge (Clemens *et al.* 1998) and for that reason can be considered a good marker of LH surge. Progesterone and prostaglandin pathways appear to act independently in the activation of downstream factors affecting ovulation (Richards *et al.* 1998). Those factors may include the factors that modify fibroblast function, chemokines that recruit inflammatory cells, and growth factors such as vascular endothelial growth factor (VEGF) that initiate angiogenesis (Richards *et al.* 1998).

The LH-induced extracellular protease ADAMTS1 is another crucial gene during ovulation. ADAMTS1 is synthesized in granulosa cells but the protein translocates to the extracellular matrix, to induce the production of versican (for a review see (Richards *et al.* 1998). The protein level is high at 8 hrs post LH and is kept elevated at least up to 16 hrs post LH. Null mice have only 10% of successful ovulation (Shindo *et al.* 2000). The role of ADAMTS1 on the activation of proteases such as metalloproteinases (MMP) has been suggested (Kuno *et al.* 1999), which could indicate a role during follicle rupture and the long-term LH response.

1.8.4.2 Long-term response to LH

Increased expression of genes involved in follicle rupture is part of the long-term response to LH. Collagen degradation and cellular death are common in the ovarian epithelium during follicle rupture and ovulation and are activated by the LH surge (Murdoch & Gottsch 2003). Two families of enzymes govern degradation of the ovarian epithelium during ovulation: metalloproteinase and plasminogen activator (Murdoch & Gottsch 2003). Expression of both of those enzyme families is induced by the LH surge. Mice with resistance to collagenase fail to ovulate and are infertile (Liu *et al.* 1995).

Tumor necrosis factor α (TNFα) induces the expression of collagenase genes (MMPs) and can also induce cellular death by initiating an inflammatory necrosis or apoptosis (Murdoch & Gottsch 2003). Pre-ovulatory follicles of rat (Rice *et al.* 1998), bovine (Zolti *et al.* 1990) and humans (Loret de Mola *et al.* 1998) secrete TNFα. Intrafollicular injection of TNFα antibodies after GnRH treatment leads to inhibition of collagen degradation and blockage of ovulation (Murdoch *et al.* 1997, Johnson *et al.* 1999). Moreover, ovarian perfusion with TNFα results in enhanced ovulation rates (Brannstrom *et al.* 1995).

LH–induced ovulation is also associated with an increase in the expression of another group of proteases called kallikreins (Bhoola *et al.* 1992, Clements *et al.* 1995). Kallikreins act in the tissue kininogens and release two bioactive kinin peptides, the kallidin and the bradykinin. Both of those peptides can bind to cell surface and mediate inflammatory effectors (Richards *et al.* 1998). This family group is gaining importance as research progresses. Bradykinin induced ovulation in infused mice ovaries (Hellberg *et al.* 1991). The use of epostane (an enzyme that blocks the conversion of pregnenolone to progesterone) also blocks the LH-induced kallikrein expression (Gao *et al.* 1992). Thus, progesterone may regulate kallikrein activity.

A recent report shows that the LH surge, as well as LH-induced TNF and IL-1, increases the expression of interleukin-8 (IL-8) (Arici *et al.* 1996), which is a potent chemoattractant (attracting neutrophils and macrophage). Expression of IL-8 is increased in follicular fluid of pre-ovulatory follicles in humans (Arici *et al.* 1996). Thus, the LH surge induces expression of IL-8, which leads to the appearance of neutrophils and macrophage close to the ovulatory follicle. Perhaps the inflammatory cells may respond to LH-induced prostaglandins inducing the secretion of protease enzymes. The protease enzymes will lead to degradation of extracellular connective tissue and ultimately follicle rupture, which is the ultimate completion of the ovulation process.

CHAPTER 2

GENERAL OBJECTIVE AND HYPOTHESIS

The general objective of this thesis research was to determine the effects of follicular aging and duration of superstimulation on oocyte competence and granulosa cell gene expression in cattle. The general hypothesis is that follicular aging and follicular superstimulation will be reflected in granulosa cell gene expression and will affect oocyte competence.

2.1 Specific Objectives

1) To evaluate oocyte competence using a superstimulation model and *in vitro* fertilization after:

- Short (4-day) & long (7-day) FSH-stimulated follicle growing phases
- A period of FSH starvation following 4 d FSH

2) To determine the effect of superstimulation treatment on gene expression of granulosa cells.

3) To determine transcriptome profile of granulosa cells exposed to short or long follicular growing phases (analogous to 3 or 2 wave estrous cycle, respectively) using a bovine superstimulation model.

4) To determine the effect of FSH starvation on gene expression of granulosa cells.

2.2 Specific Hypothesis

1) Oocyte competence will be:

- Improved by the longer growing phase
- Adversely affected by FSH starvation

2) Follicles that undergo superstimulation will have different gene expression than dominant follicles from natural cycle;

3) Extending the superstimulation protocol by 3 days will allow follicles to mature and it is beneficial to follicular health. Therefore we expect markers of maturity, cellular health and survival and also markers of post LH surge to be upregulated in the long stimulation protocol;

4) FSH starvation will induce changes in the gene expression profile when compared to a protocol with continuous FSH support. Therefore we expect markers of maturity, cellular health and survival turned off by FSH starvation.

CHAPTER 3

LENGTH OF THE FOLLICULAR GROWING PHASE AND OOCYTE COMPETENCE IN BEEF HEIFERS

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Relationship of this study to the dissertation

In Chapter 3 we evaluate the effect of the duration of the growing phase of the ovulatory follicle and a total period of 96 h of FSH starvation in oocyte competence using a superstimulation model and *in vitro* fertilization. This is chapter is of a great importance in this thesis since function as a base and rationale all other chapters. in Chapter 4, 5 and 6 we use microarrays to evaluate the effect of follicular aging and length of superstimulation protocol in the gene expression of granulosa cells. Microarray is a hypothesis generator tool, so in those chapter many hypothesis is being suggested after data analysis. Chapter 3 is the only chapter where a specific hypothesis is being tested. We tested the hypothesis that prolongation of the growing phase of the ovulatory follicle after superstimulation will improve oocyte competence, while FSH starvation will adversely affect oocyte competence. The results of this study guided us to better understanding the differently expressed gene list obtained in the following chapters.

3.1 Abstract

We tested the hypotheses that extending the duration of follicular growth by superstimulation increases oocyte competence, and that FSH starvation at the end of superstimulatory treatment decreases oocyte competence. Heifers were assigned randomly to Short FSH, FSH starvation, and Long FSH groups (n=8/group). Five to 8 d after ovulation, follicle ablation was performed, and a progesterone-releasing device (CIDR) was placed intravaginally. The Short FSH and FSH starvation groups were given eight doses of FSH im at 12 h intervals, whereas the Long FSH group was given 14 doses. Prostaglandin $F_{2\alpha}$ was administered twice (12 h apart) and CIDR removed in Day 3 (Day0=wave emergence) in the Short FSH group, and in Day 6 in the other two groups. LH was given 24 h after CIDR removal and cumulus-oocyte-complexes (COC) were collected 24 h later. The COC were matured *in vitro* for 6 h and fertilized (IVF); embryos were cultured for 10 d. A greater number of ≥9 mm follicles were detected in the Long FSH group than in the FSH starvation and Short FSH groups (25.4±5.3, 11.0±2.1, 10.6±2.3, respectively; $P<0.03$). A greater proportion of expanded COC were collected from the Long FSH than from the FSH starvation group ($P<0.001$); the Short FSH group was intermediate (93, 54 and 74%, respectively). The FSH starvation group had a greater proportion of poor quality oocytes than the Short and Long FSH groups (70, 45 and 33%, respectively; $P<0.001$) and cleavage rate was lower (22, 54 and 56%, respectively; $P=0.003$). The proportion of oocytes that developed into embryos (morulae+blastocysts in Day 9 post-IVF) was also lower in the FSH starvation group than in the Short and Long FSH groups, (5 vs 25 and 37%; $P=0.04$); the later two groups did not differ. The Long FSH treatment resulted in 2.5 and 3.4 times more transferable embryos per animal (morulae+blastocysts) at Day 9 post-IVF than the Short FSH and FSH starvation groups (5.6, 2.5, 1.7 embryos/animal respectively; $P=0.04$). In conclusion, extending the standard superstimulation protocol by 3 d enhanced the ovarian response to FSH treatment, while a period of FSH starvation after superstimulatory treatment compromised oocyte quality and fertilization process.

Keywords: follicles, FSH, growing phase, heifer, oocyte competence superstimulation.

3.2. Introduction

Most cows have two or three follicular waves during an interovulatory interval (Ginther *et al.* 1989c, Adams 1994).The luteal phase and the interovulatory interval are 2 to 3 days shorter, the duration of dominance of the first follicular wave is 2 to 3 days longer, and the interval from emergence of the final wave to ovulation is 3 days longer (9 vs 6 days) in two- wave than in three-wave cycles (Ginther *et al.* 1989a, Mihm *et al.* 2002, Jaiswal *et al.* 2009). Furthermore, the ovulatory follicle grows 3 days longer in a high progesterone environment (before luteolysis) in a two-wave cycle and ultimately attains a greater preovulatory diameter than in a three-wave cycle (Dias *et al.* 2012b). However, the period of follicle growth under a low-progesterone environment (i.e., after luteolysis) is similar between two- and three-wave cycles (i.e., 3 days) (Jaiswal *et al.* 2009). Until recently, the relationship between the number of waves in a cycle and fertility has been contradictory (Knopf *et al.* 1989, Ahmad *et al.* 1997, Townson *et al.* 2002, Bleach *et al.* 2004, Todd *et al.* 2008). However, in a recent study involving a large number of cows (n=365) (Dias *et al.* 2012b), no difference was detected in pregnancy rate between those given short-progesterone exposure during the growing phase of the ovulatory follicle (analogous to a three-wave cycle) vs those given longer progesterone exposure (analogous to a two-wave cycle). These results do not support the notion that oocytes from dominant follicles of two-wave cycles are aged and are, therefore, less fertile than those from three-wave cycles.

A superstimulation model has also been used to examine the effects of progesterone and the duration of the follicular growing phase in oocyte competence (Dias *et al.* 2012a, Garcia Guerra *et al.* 2012). Progesterone-induced prolongation of the follicular growing phase (analogous to a two-wave cycle) resulted in a greater proportion of follicles that reached ovulatory size (Dias *et al.* 2012a, Garcia Guerra *et al.* 2012).

Moreover, there was no difference between longer and shorter progesterone exposure (analogous to two- and three-wave cycles, respectively) in the developmental competence of oocytes matured and fertilized *in vivo* (Dias *et al.* 2012a).

Some have suggested that increasing the interval from the end of FSH treatments to removal of a progesterone-releasing device (i.e., a period of FSH starvation) may be beneficial for oocyte maturation (Blondin *et al.* 1997b, Sirard *et al.* 1999, Blondin *et al.* 2002). A 48-hour period of FSH starvation at the end of superstimulatory treatment was associated with an increase in the number of embryos produced *in vitro* (Blondin *et al.* 1997b). However, a period of 144 h of FSH starvation after superstimulatory treatment (under a low progesterone environment) resulted in 100% ovulation failure (Jaiswal R.S. *et al.* 2006), and 84 h of FSH starvation (luteal-phase levels of progesterone for 60 h followed by low progesterone) resulted in 50% ovulation failure (Dias *et al.* 2012a). The effects of FSH starvation in the oocyte itself could not be accessed in the later two studies.

The objective of this study was to determine the effects of the duration of the growing phase of the ovulatory follicle and a total period of 96 h of FSH starvation in oocyte competence using a superstimulation model and *in vitro* fertilization. We tested the hypothesis that prolongation of the growing phase of the ovulatory follicle after superstimulation will improve oocyte competence, while FSH starvation will adversely affect oocyte competence.

3.3. Materials and methods

3.3.1. Animals and treatments

The experiment was conducted at the Western College of Veterinary Medicine at University of Saskatchewan, during July and August. Hereford crossbred beef heifers, weighing 346.5 ± 5.8 kg (mean ± SEM), and maintained in outdoor pens, were used.

Procedures were conducted in accordance with the guidelines of the Canadian Council in Animal Care and were approved by University of Saskatchewan Protocol Review Committee. Fix paragraph spacing throughout – it is not consistent. Use indentation or separate by a line spacing.

Heifers (n=51) were given a prostaglandin $F_{2\alpha}$ analog (500 μg cloprostenol im; Estrumate, Schering-Plough Animal Health, Pointe-Claire, PQ, Canada) to synchronize estrus, and examined daily by transrectal ultrasonography using a 7.5 MHz linear-array transducer (Aloka SSD-900; Tokyo, Japan) to detect ovulation. The first 24 heifers that ovulated after prostaglandin treatment were used in the study. Transvaginal ultrasound-guided ablation of follicles ≥5 mm in diameter was done 5 to 8 days after ovulation to synchronize emergence of a new follicular wave (Bergfelt DR *et al.* 1994). A progesterone-releasing device (CIDR-B; Pfizer Canada Inc., Saint-Laurent, QC, Canada) was placed in the vagina immediately after follicle ablation. The day of emergence of the new follicular wave (Day 0) was considered to be the day after ablation (Bergfelt DR *et al.* 1994). Heifers were assigned randomly to three groups to be superstimulated (n=8 per group; Fig. 3.1): 1) Short FSH, 2) FSH starvation, and 3) Long FSH. Starting in Day 0, the Short FSH was given 8 im doses of FSH (Folltropin-V; Bioniche Animal Health, Belleville ON, Canada) at 12 h intervals over 4 d (Day 0.5 to 4; each dose equivalent to 25 mg of NIH-FSH-P1), and two luteolytic im doses of prostaglandin (500 μg of cloprostenol) 12 h apart in Day 3. The FSH starvation group was given similar treatment of eight doses of FSH; however, the two luteolytic doses of prostaglandin were given in Day 6. The Long FSH group was given FSH every 12 h for 7 d (i.e., 14 doses, each dose equivalent to 25 mg of NIH-FSH-P1; Day 0.5 to 7); two doses of prostaglandin were given in Day 6. The CIDR were removed concurrent with the second prostaglandin treatment (Day 3.5 in short FSH group, and Day 6.5 in FSH starvation and long FSH groups). Heifers were given 12.5 mg pLH im (Lutropin-V, Bioniche Animal Health) 24 h after CIDR removal, and follicular aspiration was performed 24 h after pLH treatment. Thus, the gonadotropin-free period (interval between last FSH and pLH injections) was 12 h in short and long FSH groups and 84 h in FSH starvation group. The ovaries of each heifer were examined by transrectal ultrasonography 12 h after pLH treatment to determine the number and size of follicles.

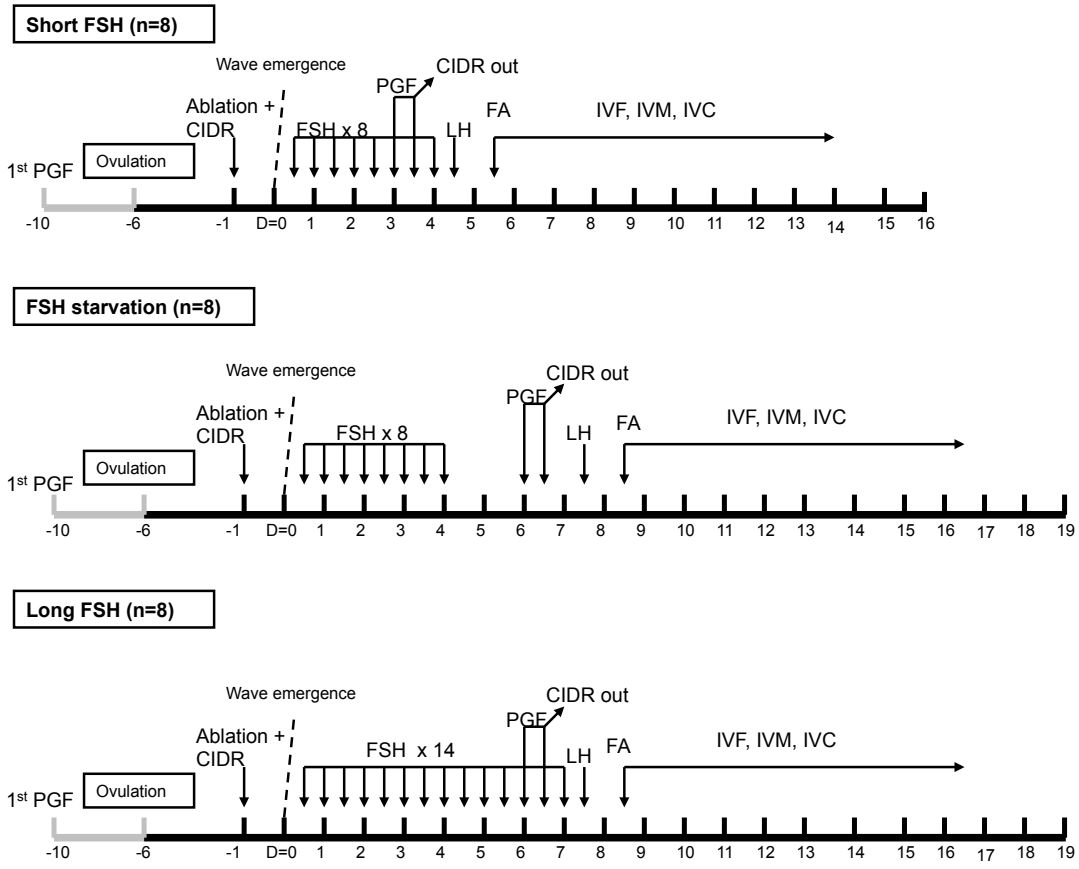

Fig. 3.1 Experimental protocol used to test the effect of the duration of the follicular growing phase and FSH starvation in oocyte competence after *in-vitro* fertilization in superstimulated heifers. Five to 8 days after ovulation, follicles ≥5 mm were ablated and a progesterone-releasing device (CIDR) was placed intravaginally. FSH treatment was started in the day of wave emergence (Day 0). Short FSH and FSH starvation groups were given 8 FSH treatments, whereas the Long FSH group was given 14 FSH treatments. Two doses of prostaglandin (PGF) was given in Day 3 in the Short FSH group and in Day 6 in the other groups. CIDR were removed at the time of second PGF treatment, and pLH was given 24 h later. Follicular aspiration (FA) for oocyte collection was performed 24 h after LH treatment.

3.3.2. Oocyte collection and classification

Caudal epidural anesthesia was induced (2% lidocaine HCL and epinephrine USP; Bimeda-MTC Animal Health Inc., Lavaltrie, QC, Canada) and cumulus-oocyte complexes (COC) were collected by transvaginal ultrasound-guided follicular aspiration using a 7.5 MHz convex-array transducer. Follicular contents were aspirated using a vacuum pump with a flow-rate of 20-25 mL/min (Vacuum pump: Allied Healthcare Products, Inc, St Louis, MO, USA) into a 70-micron embryo filter (Emcon filters; Veterinary Concepts; Spring Valley, WI, USA) containing Dulbecco's phosphate buffered saline (dPBS, Invitrogen Inc., Burlington, ON, Canada) with ET Surfactant (0.3% Pluronate; Bioniche Animal Health) and sodium heparin (2 IU/mL). Collection of COC was scheduled over a 12-day period such that one heifer from each group was represented in each collection day (i.e., 3 heifers per collection day). The COC were transported from barn to the laboratory in dPBS plus 5% newborn calf serum in a portable incubator set at 37°C.

The COC were identified by stereomicroscopy at 10X magnification, washed three times in 1X dPBS (with 5% newborn calf serum) and graded based on the status of cumulus cells surrounding the oocyte (i.e., compact, partially expanded, or expanded). They were further graded based on the number of layers of cumulus cells and homogeneity of ooplasm (see supplementary information).

3.3.3. In-vitro maturation, fertilization and culture (IVM, IVF, and IVC)

The COC were washed three times in maturation medium consisting of TCM-199 supplemented with 5% CS, 5 μg/mL LH (Bioniche, Belleville, ON, Canada), 0.5 μg/mL FSH (Bioniche) and 0.05 μg/mL gentamicin (Prentice *et al.* 2011). To manage an uneven number of COC collected per animal, 4 to 6 COC were placed in droplets (5 μL/COC) of maturation medium (under mineral oil) and incubated for 6 h (38.5 °C, 5% CO_2 in air and high humidity) for *in vitro* maturation (see supplementary information). COC were then washed three times in Brackett-Oliphant medium (Parrish *et al.* 1995) supplemented with 10% bovine serum albumen. Frozen-thawed pooled semen from three fertile Holstein

bulls (2 straws per bull) was washed through Percoll gradient (45% and 90%) (Parrish *et al.* 1995), and diluted in fertilization (Brackett-Oliphant) medium (Brackett & Oliphant 1975) to a final concentration of 3×10^6/mL for *in vitro* fertilization (IVF). For IVF, 4 to 6 COC were placed in droplets (5 µL/COC) of sperm in Brackett-Oliphant medium (under mineral oil) and incubated for 18 h (38.5°C, 5% CO_2 in air and high humidity). Following IVF, cumulus cells and sperm were removed by pipetting and oocytes were washed three times with CR1aa medium (*in vitro* culture medium) (Prentice *et al.* 2011). The presumptive zygotes (4 to 6) were then placed in droplets of *in vitro* culture medium (5 µL/zygote) under mineral oil, and incubated at 38.5°C under 5% CO_2, 90% N_2, 5% O_2 and high humidity. The cleavage rates were recorded 2 days after the end of IVF and blastocyst rates, at day 7, 9 and 10 post-IVF. Embryo development was evaluated (Stringfellow & Seidel 1998) based on the criteria of the International Embryo Transfer Society (IETS).

3.3.4. Statistical analyses

The follicular data were arranged in different size categories: ≤5mm, 6-8mm, and ≥9mm. Dependent variables were analyzed by one-way analysis of variance, using a general linear model procedure (GLM; SAS Learning Edition 4.1; SAS Institute Inc., Cary, NC, USA). Normality of residuals was tested by the Shapiro-Wilk test. Probability values >0.05 were considered non significant. Tukey's post-hoc tests were performed if main effects were significant ($P \leq 0.05$). The values are expressed as mean ± SEM. Proportional data were compared using logistic regression. The percentage of COC was also analyzed in a per-animal basis using a 3x3 factorial design (compact, partially expanded and expanded vs Short FSH, Long FSH and FSH starvation) after transforming the data to the arcsine.

3.4 Results

One heifer in Long-FSH group had several ovulations prior to COC collection (newly formed CL were detected by ultrasonography) and was excluded from the follicular data analysis. The few COC collected from this heifer were, however, included in the embryo development data.

3.4.1 Follicular response and COC collection

A greater total number of follicles, and a greater number of large follicles (≥9mm) were detected in the Long FSH group than in the Short FSH or FSH starvation groups ($P<0.05$; Table 3.1). The COC collection efficiency (number of COC recovered/ number of follicles aspirated x 100) did not differ among groups (Table 3.1); the combined collection efficiency was 52%. The number of COC recovered per animal tended to be greater in the Long FSH group ($P=0.1$) as a result of a numerically greater number of follicles aspirated (Table 3.1) vs other groups.

Table 3.1. Ovarian response and cumulus-oocyte-complex (COC) collection efficiency (mean ± SEM) in heifers superstimulated with FSH for a short duration (4 d), a short duration (4 d) followed by a period of FSH starvation (3.5 d), or a long duration (7 d) before being given pLH to induce in vivo oocyte maturation.

Endpoints recorded at 12 hr after LH treatment	Short FSH	FSH Starvation	Long FSH
Number of heifers	8	8	7
Total number of follicles (≥3 mm)	18.0±4.2[a]	19.6±2.7[a]	34.7±5.8[b]
Number of follicles ≥9 mm	10.6±2.3[a]	11.0±2.1[a]	25.4±5.3[b]
Number of follicles 6-8 mm	5.0±1.8	7.0±1.9	8.3±1.6
Number of follicles 3-5 mm	2.4±0.8	1.6±0.3	1.0±0.4
Number of follicles aspirated per animal	17.5±4.7	20.7±3.1	28.8±8.1
COC recovered per animal*	10.5±2.4	9.25±2.0	17.8±3.7
Number of COC collected/follicle aspirated (%)	85/144 (59.0)	74/157 (47.1)	125/243 (51.4)

[ab] Within rows, values with different superscripts are different (P<0.05)

* Values tended to differ (P=0.1)

3.4.2. COC evaluation

Data in a per animal basis are presented in Fig. 3.2, while group percentages are summarized in Table 3.2. More than 90% of COC collected in the Long FSH group were expanded. The number of expanded COC was lower in the FSH starvation group than in the other two groups (P<0.001). The FSH starvation group had a greater number of low-quality (P<0.001) and partially expanded COC (P<0.001) than the Short and Long FSH groups. The number of compact COC was greater in the Short FSH group than in the Long FSH group (P=0.05), and tended to differ from the FSH starvation group (P=0.1). The prevalence of poor quality COC (Grade 3 and 4 combined) was greater (P<0.001) in the FSH starvation group than in the Long and Short FSH groups; i.e., 52/74 (70.3%), 42/127 (33.1%), and 38/85 (44.7%), respectively.

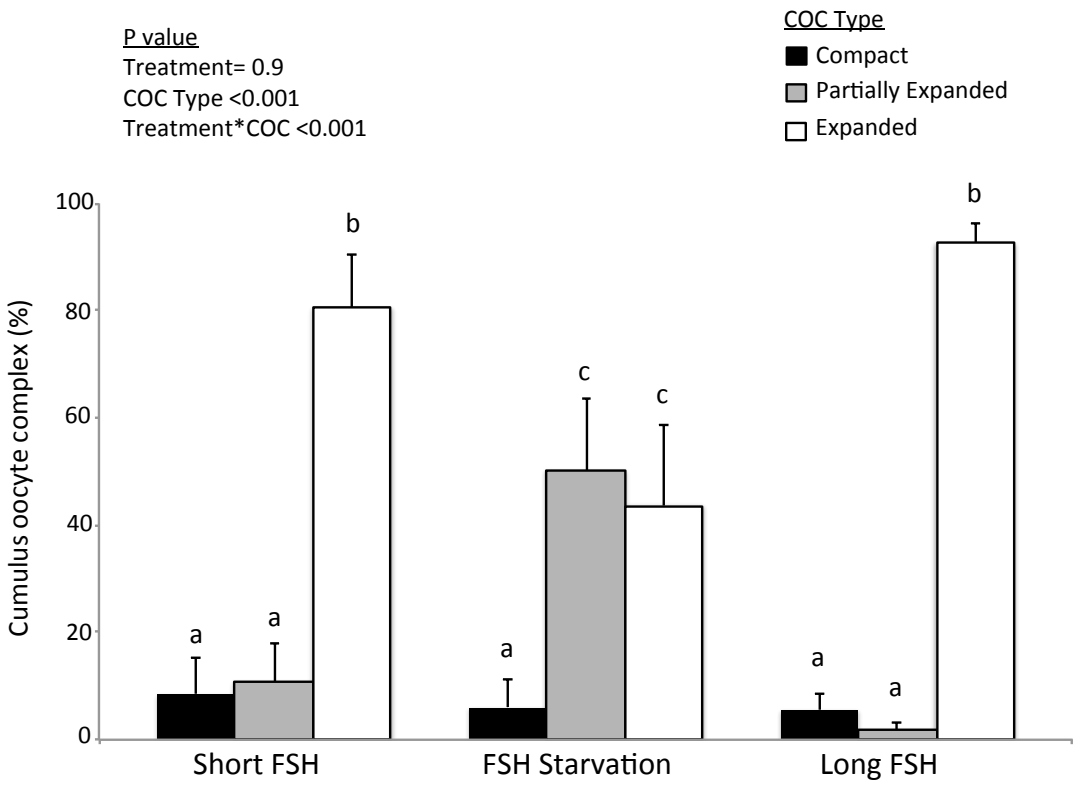

Fig. 3.2 Percentage of cumulus-oocyte complexes (COC) with either compact, partially expanded, or expanded cumulus cells after superstimulatory treatment with FSH for a short duration (4 d), a short duration (4 d) followed by a period of FSH starvation (3.5 d), or a long duration (7 d) before being given pLH to induce in vivo oocyte maturation in heifers (n=8 per group).

[abc] Values with no common superscript are different (P<0.05).

Table 3.2. Proportions (%) of cumulus-oocyte complexes collected from heifers after superstimulatory treatment with FSH for a short duration (4 d), a short duration (4 d) followed by a period of FSH starvation (3.5 d), or a long duration (7 d) before being given pLH to induce in vivo oocyte maturation (n=8 per group).

	Short FSH	FSH Starvation	Long FSH
Expanded	63/85 (74.1)[a]	40/74 (54.1)[b]	118/127 (92.9)[a]
Grade 1 and 2	36/85 (42.3)[a]	16/74 (21.6)[b]	77/127 (60.6)[a]

Grade 3 and 4	27/85 (31.8)	24/74 (32.4)	41/127 (32.3)
Partially expanded	9/85 (10.6)[a]	28/74 (37.8)[b]	2/127 (1.6)[a]
Grade 1 and 2	6/85 (7.0)	4/74 (5.4)	1/127 (0.8)
Grade 3 and 4	3/85 (3.5)[a]	24/74 (32.4)[b]	1/127 (0.8)[a]
Compact	13/85 (15.3)[a*]	6/74 (8.1)[ab*]	7/127 (5.5)[b]
Grade 1 and 2	5/85 (5.9)	2/74 (2.7)	7/127 (5.5)
Grade 3 and 4	8/85 (9.4)	4/74 (5.4)	0/127 (0)

[ab] Within rows, values with different superscripts are different (P<0.05)

* Values tended to differ (P=0.1)

3.4.3. Embryo development

The cleavage rate, defined as the percentage of embryos with 2 to 8 cells at 48 h post-IVF, was lower in the FSH starvation group than in the Short- and Long-FSH groups (P=0.003; Table 3.3). No difference in cleavage rate was detected between Short and Long FSH groups. By Day 9, more morulae and blastocysts had developed in the Long FSH group compared to FSH starvation group but did not differ from the Short FSH group (P<0.04). However, when data were examined in a per animal basis, the Long FSH group produced 2.5 times more transferable embryos (morulae+blastocysts at Day 9) than the Short FSH group and 3.4 times more transferable embryos than the FSH starvation group (P=0.04; Table 3.3). The difference in blastocyst rate in either Day 9 or 10 did not reach significance (Table 3.3). The Long FSH group had a higher proportion of cleaved embryos (2-8 cell stages at 48 h post-IVF) reaching morula+blastocyst stages in Day 9 compared to the short FSH group (45/69 versus 20/44; chi-square P=0.03).

One heifer in the FSH starvation group was suspected to be an outlier since proportional embryo development was greater in this heifer than the average for the remainder of the group: Cleavage, 13/17 vs 12/55 (P<0.001); 8-cell embryos at 48 h post-IVF, 8/17 vs 2/55 (P<0.001); Morulae + blastocysts at Days 7 and 9, and blastocyst rate at Days 9 and 10 (P<0.001 for all four endpoints). Analyses were repeated after removal

of data from this heifer (Table 3.4). Previously observed differences among groups were more apparent when the outlier was excluded from analyses. The cleavage and blastocyst rates were lower in the FSH starvation group than in the Short and Long FSH groups ($P<0.01$; Table 3.4). The FSH starvation group had markedly lower morula and blastocyst rates in Day 7 and Day 9 ($P=0.008$ and $P=0.001$, respectively; Table 3.4) and a lower blastocyst rate in Day 9 and Day 10 post-IVF ($P=0.02$ and 0.03, respectively; Table 3.4).

Table 3.3. Cleavage rate and embryo development after in vitro fertilization of oocytes [proportion, (%)] from heifers after superstimulatory treatment with FSH for a short duration (4 d), a short duration (4 d) followed by a period of FSH starvation (3.5 d), or a long duration (7 d) before being given LH to induce in vivo oocyte maturation (n=8 per group).

	Short FSH	FSH Starvation	Long FSH
Number of heifers	8	8	8
Cleavage rate at 48 h post-IVF			
1 cell	37/81 (45.7)[a]	47/72 (65.3)[b]	54/123 (43.9)[a]
2-8 cells	44/81 (54.3)[a]	25/72 (34.7)[b]	69/123 (56.1)[a]
>8 cells	15/81 (18.5)	10/72 (13.9)[*]	32/123 (26.0)
Morulae + blastocysts at Day 7 post-IVF	17/81 (21.0)	12/72 (16.7)	27/123 (21.9)
Morulae + blastocysts at Day 9 post-IVF (transferable embryos)	20/81 (24.7) [ab]	13/72 (18.1)[a]	45/123 (36.6)[b]
Transferable embryos per heifer at Day 9 post-IVF	20/8 (2.5)[a]	13/8 (1.7)[b]	45/8 (5.8)[a]
Blastocysts at Day 9 post-IVF	11/81 (13.6)	10/72 (13.9)	26/123(21.1)
Blastocysts at Day 10 post-IVF	11/81 (13.6)	12/72 (16.7)	28/123 (22.8)
Expanded + hatched blastocysts at 10 days	10/81 (12.3)	7/72 (9.7)	17/123 (13.8)

[ab] Within rows, values with different superscripts are different ($P<0.05$)

*FSH starvation group tended to have fewer >8-cell embryos than other groups (P=0.1).

Table 3.4. Embryonic development after in vitro fertilization of oocytes collected from heifers treated with FSH for a short duration (4 d), a short duration (4 d) followed by a period of FSH starvation (3.5 d), or a long duration (7 d) before being given pLH to induce in vivo oocyte maturation. One heifer from the FSH starvation group was considered an outlier, and her embryos are not included in the table.

	Short FSH	FSH Starvation	Long FSH
Number of heifers	8	7	8
Cleavage rate 48 h post-IVF (%)	44/81 (54.3)[a]	12/55 (21.8)[b]	69/123 (56.1)[a]
Morulae+blastocysts in Day 7 post-IVF (%)	17/81 (21)[a]	2/55 (3.6)[b]	27/123 (21.9)[a]
Morulae+blastocysts in Day 9 post-IVF (%)	20/81 (24.7)[a]	3/55 (5.4)[b]	45/123 (36.6)[a]
Blastocysts in Day 9 post-IVF (%)	11/81 (13.6)[a]	1/55 (1.8)[b]	26/123 (21.1)[a]
Blastocysts in Day 10 post-IVF (%)	11/81 (13.6)[a]	3/55 (5.4)[b]	28/123 (22.8)[a]

[ab] Within rows, values with different superscripts are different (P<0.05)

3.4. Discussion

Results of the present study document that an extended period of FSH support improved the superstimulatory response by extending the follicular growing phase and permitting more follicles of the cohort to reach an ovulatory size. Extending the follicular growing phase was also associated with recovery of more fully expanded COC. Despite the extended period of FSH support (Long FSH group) resulting in markedly more embryos in each successive stage (cleavage, morula, blastocyst) than conventional

treatment (Short FSH group), differences in proportional development were not significant.

Increasing the interval from the end of FSH treatment to the time of pLH treatment (FSH starvation) resulted in poor quality oocytes and fertilization failure. Moreover, of the few oocytes that cleaved after fertilization in the FSH starvation group, proportionally fewer developed to the morula and blastocyst stages after 9 days of *in vitro* culture than in the other two groups. These results are consistent with the stated hypothesis that FSH starvation decreases oocyte competence.

One of the major limitations of superstimulation treatment is the variability in ovarian response (Adams *et al.* 1994, Mapletoft *et al.* 2002). In one study (Looney 1986), 30% of 2048 cows produced 70% of the embryos, whereas 24% cows failed to produce any viable embryos. Major determinants of the superstimulatory response in cattle are the intrinsic number of follicles present at the time of wave emergence in a given individual (Singh *et al.* 2004), and when FSH treatment is initiated in relation to wave emergence (Nasser *et al.* 1993). Superstimulatory treatment initiated more than one day before or after wave emergence is associated with a lower ovulatory response, and the response is highly correlated with the number of follicles present at the time of wave emergence. In the present study, extending the superstimulation protocol by 3 days resulted in a greater number of large follicles at the end of treatment, a result that is consistent with that of other studies (Dias *et al.* 2012a, Garcia Guerra *et al.* 2012). It remains unknown whether the improved ovarian response following an extended superstimulation protocol is attributed to i) attainment of a greater number of follicles within the wave to an ovulatory size, or ii) recruitment of additional follicles into the wave.

As in a previous report (Dias *et al.* 2012a), the amount of FSH given every 12 h was maintained constant among groups (25 mg every 12 h) in this study; therefore, the total FSH dose used in this study varied among groups. The Long FSH group was given a total dose of 350 mg while Short FSH and FSH starvation groups were given 200 mg of FSH. The increased ovarian response in the Long FSH group may be attributed to the higher total FSH dose used. However, a greater ovarian response was also observed with

an extended (7-day) superstimulation protocol using the same total dose of FSH as for a 4-day protocol (Garcia Guerra *et al.* 2012), suggesting that extra time and not the total dose of FSH is responsible for the increased ovulatory response after superstimulation.

In previous studies, FSH starvation resulted in a loss of ovulatory capability in 100% of follicles that developed in a low progesterone environment (Jaiswal R.S. *et al.* 2006), and in about 50% of follicles that developed under luteal-phase levels of progesterone (Dias *et al.* 2012a); however, oocyte competence was not assessed. The present study was designed specifically to examine the effects in oocyte competence; hence, ovulatory capability was not assessed because COC were collected before ovulation took place. Follicles from the FSH starvation group had a follicular growing phase that was of similar length to that of the Long FSH group; however, most of the COC from the FSH starvation group were not fully expanded, and the developmental capacity of oocytes from this group was clearly compromised. Therefore, FSH starvation after superstimulation is associated with a loss of both ovulatory capability and oocyte competence. Others have suggested a beneficial effect of FSH starvation ("coasting") in oocyte competence after superstimulation (Blondin *et al.* 1997b, Sirard *et al.* 1999, Blondin *et al.* 2002). A greater proportion of oocytes developed into embryos *in vitro* after a 48-hour period of FSH starvation compared to 24 and 72 h (Blondin *et al.* 1997b). Contradictory, 48 h of FSH starvation did not improve oocyte competence of Bos indicus oocytes (Monteiro *et al.* 2010). In a more recent study (Nivet *et al.* 2012), 92 h of FSH starvation resulted in an increase in the number of ≥10 mm follicles, but blastocyst formation rate was reduced compared to 44 and 68 h. In the present study, follicles in the FSH starvation group were exposed to an 84-hour gonadotropin-free period (60 h under high progesterone and 24 h under a low progesterone) before pLH treatment (i.e., 96 h of FSH starvation). Failure to cleave occurred in 95% of oocytes from the FSH starvation group, only 3 (out of 55) transferable embryos were obtained from this group. Fertilization failure could be a reason for the reduced cleavage rates. However, whether sperm penetration occurred in these oocytes remains unknown. In the few embryos obtained in the FSH starvation group, development was also compromised since proportionally fewer developed to the morula and blastocyst stages at Day 9. Based on

the present and previous results from our lab (Jaiswal R.S. *et al.* 2006, Dias *et al.* 2012a), and others (Nivet *et al.* 2012), 96 h of FSH starvation results in atretic follicles that are incapable of ovulating and these follicles would appear to contain atretic oocytes.

The relationship between the wave pattern during the estrous cycle (i.e., 2-wave vs 3-wave cycle) and fertility has been an issue of long-standing controversy (Ahmad *et al.* 1997, Townson *et al.* 2002) (Bleach *et al.* 2004). In the present study, the Long FSH group was a superstimulated model of a 2-wave cycle in that it created a long growing phase of the ovulatory follicle; the Short FSH group was a superstimulated model representative of a 3-wave cycle in that it created a short growing phase. Proportion of oocytes that developed to the blastocyst stage did not differ between groups, but the mean number of transferable embryos obtained per animal was greater in the Long FSH group than in the Short FSH group. This is consistent with our previous study involving *in vivo* fertilization (Dias *et al.* 2012a), and another recent study (Garcia Guerra *et al.* 2012) in which a numerically greater number of transferable embryos was collected after the use of an extended (7-d) superstimulation protocol compared to a standard (4-d) protocol, though there was insufficient statistical power to show a statistical difference. Another recent study (Dias *et al.* 2012b) in a large group of cows (n=365) documented that short progesterone exposure during the growing phase of the ovulatory follicle (analogous to a 3-wave cycle) was not associated with higher fertility than long progesterone exposure (analogous to a 2-wave cycle). The combined results from these studies contradict the long-standing notion that oocytes from dominant follicles of 3-wave cycles are more fertile than those from 2-wave cycles.

In conclusion, extending the period of FSH treatment during superstimulation resulted in prolongation of follicular growth, a greater number of large follicles available for oocyte collection and in 2.5 times more transferable embryos per heifer. Follicular maturation resulting from an extended period of FSH support was associated with collection of more COC that were fully expanded; however, numbers were insufficient to adequately test the hypothesis of an effect in developmental competence. The hypothesis that a total of 96 h of FSH starvation (84 h of gonadotropin free + 12 h of LH) will

adversely affect oocyte competence was supported; i.e., FSH starvation was associated with collection of low quality oocytes with severely retarded fertililization potential and developmental competence.

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3.5 Supplementary information

Cumulus-oocyte-complexes (COC) were evaluated based on the appearance of cumulus cells and the ooplasm. Cumulus cells were defined as compact, partially expanded and expanded. The COC with >5 layers of cumulus cells and homogeneous brownish ooplasm were classified as Grade 1. Those with <5 intact layers of cumulus cells and homogeneous or slightly non-homogeneous areas in the ooplasm were classified as Grade 2. The COC with intact cumulus layers but distinct heterogeneous ooplasm were classified as Grade 3. While COC with very light or very dark brown/blackish ooplasm were classified as Grade 4. Denuded COC were considered Grade 4. Grade 1 and 2 were considered 'Good' and combined together for analysis of compact, partially expanded or expanded, while Grade 3 and 4 were defined as 'Bad'. The time interval from collection to placement in culture was less than 90 minutes. The amount of culture medium and number of COC per drop are summarized in Table 3.5.

Table 3.5. A representations of how the total numbers of cumulus-oocyte-complexes (COC) collected per animal was distributed per drop and the volume of culture medium used for each drop during *in vitro* culture. Fix this table …

Number of COC collected per animal	**Drop 1**		**Drop 2**		**Drop 3**		**Drop 4**	
	Number of COC in the drop	Volume of the drop (µl)	Number of COC in the drop	Volume of the drop (µl)	Number of COC in the drop	Volume of the drop (µl)	Number of COC in the drop	Volume of the drop (µl)
1	1	5						
2	2	10						
3	3	15						
4	4	20						
5	5	25						
6	6	30						
7	4	20	3	15				

8	4	20	4	20				
9	4	20	5	25				
10	5	25	5	25				
11	5	25	6	30				
12	6	30	6	30				
13	5	25	5	25	3	15		
14	5	25	5	25	4	20		
15	5	25	5	25	5	25		
16	5	25	5	25	6	30		
17	5	25	6	30	6	30		
18	6	30	6	30	6	30		
19	5	25	5	25	5	25	4	20
20	5	25	5	25	5	25	5	25

CHAPTER 4

DIFFERENTIAL GENE EXPRESSION OF GRANULOSA CELLS AFTER OVARIAN SUPERSTIMULATION IN BEEF CATTLE.

Dias F.C.F., Khan M.I.R., Sirard M.A., Adams G. P., Singh J.

Relationship of this study to the dissertation

The focus of this thesis is to determine the effects of follicular aging and duration of superstimulation on oocyte competence and granulosa cell gene expression in cattle. This objective was addressed in four different chapters (Chapters 3, 4, 5 and 6). In Chapter 3 *in vitro* production was used to evaluate oocyte competence. Granulosa cell gene expression was evaluated in Chapters 4 (the present one), 5 and 6. There was a need to first compare the gene profile of follicles after superstimulation, using a standard 4 days protocol, to dominant follicles from natural cycle. Only after studying the effect of superstimulation itself I felt confident to compare different superstimulation protocols (as in Chapters 5 and 6).

4.1 Abstract

Microarray was used to compare the gene expression of granulosa cells from dominant follicle with those after superstimulatory treatment. Cows were allocated randomly to two groups (superstimulation and control, n=6/group). A new follicular wave was induced by ablation of follicles ≥5 mm in diameter, and progesterone-releasing device (CIDR) was placed in vagina. The superstimulation group was given eight doses of 25 mg FSH at 12-h intervals starting from the day of wave emergence (Day 0), whereas the control group was not given FSH treatment. Both groups were given prostaglandin $F_{2\alpha}$ twice, 12 h apart, in Day 3 and the CIDR was removed at the second injection; 25 mg pLH was given 24 h after CIDR removal, and cows were ovariectomized 24 h later. Granulosa cells were collected for RNA extraction, amplification and microarray hybridization. A total of 190 genes were down-regulated and 280 genes were upregulated. To validate the microarray results, five genes were selected for real time-PCR (*NTS, FOS, THBS1, FN1* and *IGF2*). Expression of four genes increased significantly in the 3 different animals tested (*NTS, FOS, THBS1* and *FN1*). The upregulated genes are related to matrix remodeling (i.e. tissue proliferation), disturbance of the angiogenesis, apoptosis and oxidative stress response. We conclude that superstimulation treatment 1) results in granulosa cells that lag behind in maturation and differentiation (most of the upregulated genes are markers of the follicular growing stage), 2) activates genes involved with the *NRF2* oxidative stress response and endoplasmic reticulum stress response, and 3) disturbs angiogenesis.

Keywords : cattle, FSH, follicle, granulosa cells, genes, genomics, LH, micro-array analysis, ovary, superstimulation.

4.2 Introduction

The primary action of follicle stimulating hormone (FSH) in the female is to promote follicular development (Adams *et al.* 1992b). A group of follicles (wave or

cohort) emerges after an increase in peripheral FSH concentrations (Adams *et al.* 1992b, Jaiswal *et al.* 2004, Adams *et al.* 2008). Follicular products from the growing cohort, particularly the dominant follicle, are responsible for suppressing FSH concentrations (Adams 1994, Berfelt *et al.* 1994). When circulating FSH begins to decline, subordinate follicles stop growing and become atretic (Adams *et al.* 1993b, Adams *et al.* 1993a, Bergfelt DR *et al.* 1994, Ginther *et al.* 1999). However, the dominant follicle acquires LH receptors in its granulosa cells (Adams 1994) and is therefore no longer dependent in FSH (Bao & Garverick 1998, Ginther *et al.* 1999). Maintenance of elevated circulating concentrations of FSH rescues the subordinate follicles within the cohort from regression and thereby delays or prevents selection of a single dominant follicle; i.e., results in multiple dominant follicles capable of ovulating (superstimulatory/ superovulatory response) (Scanlon *et al.* 1968, Wildt *et al.* 1975, Adams *et al.* 1993b, Mapletoft *et al.* 2002).

Superstimulation is a technique that has been used widely in animal breeding programs, both for commercial and research proposes. One of the major limitations of superovulation is the extreme variability in the response to treatments. In one study (Looney 1986), 30% of 2048 cows produced 70% of the total embryos collected, whereas 24% of cows failed to produce an embryo. Major determinants of the superstimulatory response are the number of follicles available at wave emergence (Singh *et al.* 2004) and the timing of the onset of treatments in relation to wave emergence (Nasser *et al.* 1993, Adams *et al.* 1994, Adams 1998). The reason for the high variation among individuals in the number of follicles recruited into a wave and the proportion of follicles that fail to ovulate after superovulatory treatment remains unknown. The effect of superstimulation treatment in the follicular environment and oocyte quality is also not well understood. Previous studies suggest that superstimulation can trigger genes related to the oxidative stress response of embryos from mice (Rossignol *et al.* 2006, Fauque *et al.* 2007, Sato *et al.* 2007) and cattle (Mundim *et al.* 2009). However these studies involved analysis of the genetic status of embryos and tested only a few genes. Microarray technology allows a better understanding of molecular status by evaluating the expression levels of thousands of genes at the same time.

Current parameters used for determining oocyte competence are based on oocyte morphology, and the ability to fertilize and to develop to the blastocyst stage. However, the molecular status of follicles and oocytes subsequent to superstimulation is not known. Therefore, there is a need to determine how gene expression of a follicle is affected by preventing follicular selection and if alterations in intracellular molecular pathways can explain the extensive individual variability in the response to superstimulation treatment.

The objective was to determine the effect of superstimulation treatment in major molecular and cellular pathways, as evidenced by gene expression of granulosa cells. We tested the hypothesis that, given an equivalent growing phase, the molecular pathways related to cell differentiation are altered in granulosa cells from superstimulated follicles compared to those of single (unstimulated) preovulatory follicles.

4.3 Material and Methods

4.3.1 Animals and Treatments

The experiment was conducted on 12 cross-bred beef cows, weighing 515 to 795 kg, maintained in outdoor pens at the University of Saskatchewan during October to December. Procedures were conducted in accordance with the guidelines of the Canadian Council on Animal Care and were approved by University of Saskatchewan Protocol Review Committee.

To synchronize estrus and ovulation, the cows were given two luteolytic doses of prostaglandin ($PGF_{2\alpha}$; 500 μg of cloprostenol im; Estrumate, Schering-Plough Animal Health, Pointe-Claire, QC, Canada) 14 days apart. Emergence of a new follicular wave was synchronized by transvaginal ultrasound-guided ablation of follicles ≥5 mm in diameter 5 to 8 days after ovulation. A new follicular wave was expected to emerge 1 day after ablation (Bergfelt DR *et al.* 1994). An intravaginal, progesterone-releasing device

(CIDR-B, Pfizer Canada Inc., City, QC, Canada) was placed in the vagina immediately after follicle ablation. The cows were then allocated randomly to two groups: a) Superstimulation group (n=6), and b) Control group (no superstimulation, n=6; Fig. 4.1). Starting 1 day after follicle ablation, i.e., in the day of wave emergence (Day 0), cows in the superstimulation group were administered 8 doses of FSH im (Folltropin-V; Bioniche Animal Health, Belleville ON, Canada; each equivalent to 25 mg of NIH-FSH-P1) at 12-hour intervals over 4 days. The Control group was not given any FSH treatment. on Day 3, cows in both groups were given 2 im doses of 25 mg of $PGF_2\alpha$ 12-hours apart, and the CIDR was removed at the time of the second $PGF_2\alpha$ treatment. Cows were given 25 mg pLH im (Lutropin-V, Bioniche Animal Health) 24 hours after CIDR removal and were ovariectomized 24 hours after pLH treatment.

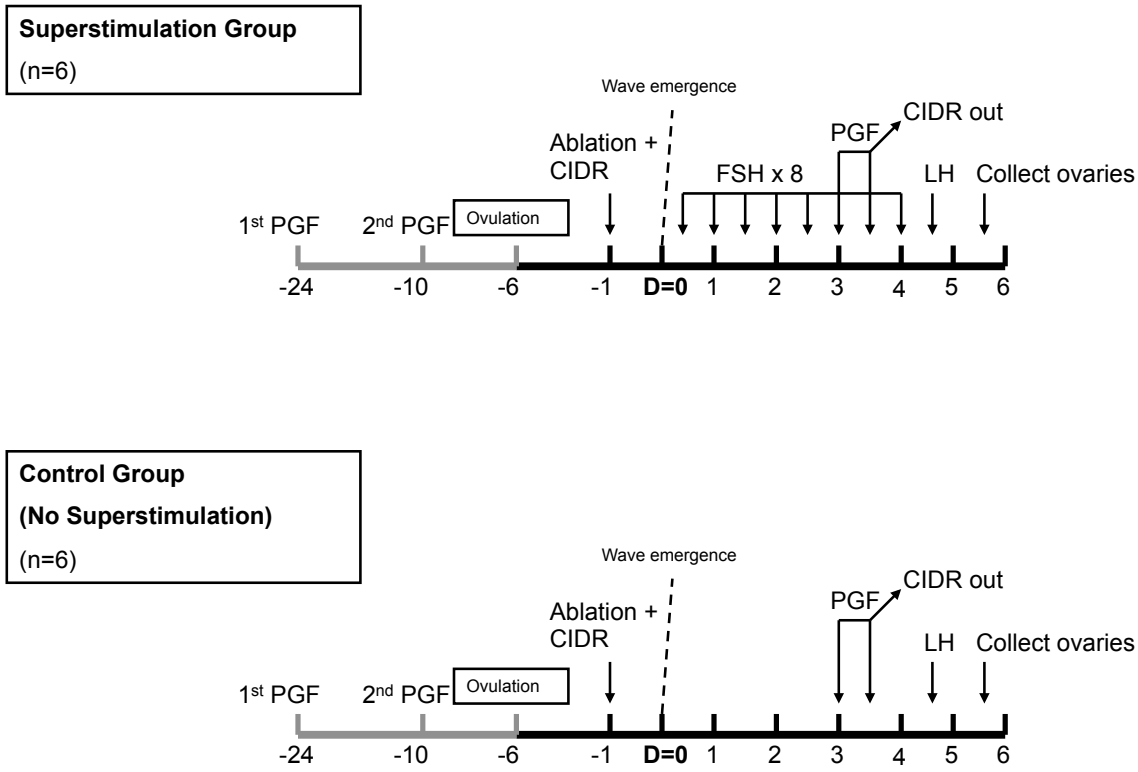


Figure 4.1. Experimental protocol used to test the effect of ovarian superstimulation on gene expression in granulosa cells. Five to 8 days after ovulation, follicles ≥5 mm were ablated and a progesterone-releasing device (CIDR) was placed intravaginally. FSH treatment was initiated at wave emergence (Day 0). Cows in the superstimulation group were given 8 doses of FSH at 12-hr intervals, and cows in the control group were not treated with FSH. on Day 3, cows in both groups were given $PGF_2\alpha$ and the CIDR was removed. LH was given 24 hours after CIDR removal, and cows were ovariectomized 24 hours later.

4.3.2 Tissue Collection

Ovariectomies were performed using a colpotomy approach, as described (Singh *et al.* 1998). Briefly, caudal epidural anesthesia was induced with 5 to 10 ml of lidocaine (Lidocaine HCL 2%, Catalog# 1LID009P, Bimeda-MTC Animal Health Inc., Lavaltrie, QC, Canada). The perineum was disinfected using an iodine-based detergent and solution. A small incision was made in the dorso-lateral aspect of the vaginal fornix. The peritoneum was ruptured manually, allowing direct access to and palpation of the reproductive tract. Local anesthesia was manually applied to the ovarian pedicle using an gauze soaked with lidocaine. A plastic clip was applied to the ovarian pedicle to minimize hemorrhage. The chain of an ecraseur was looped around the ovarian pedicle and slowly tightened until the ovarian attachments were severed. The ovaries were placed in polyethylene bags, kept on ice, and transported to the laboratory within 5 minutes after collection. The number of follicles in both ovaries was counted and the dominant follicle (control group) or the three largest follicles (superstimulated group) were identified by visual assessment and confirmed by measuring the diameter after follicles were opened. The goal was to collect antral and mural granulosa cells. Antral granulosa cells were those floating freely in the follicular fluid, while the mural granulosa cells were those that were attached to the inner wall of the follicle. Antral granulosa cells were collected by aspiration of the follicular antrum using a 20 gauge needle and syringe. Follicles were flushed 3 times with Dulbecco's phosphate buffer saline (dPBS, Invitrogen Corporation, catalog 14190-144, Burlington, ON, Canada). The cumulus-oocyte-complex (COC) was then identified and separated from the aspirate. The follicular fluid was centrifuged and the pellet of antral granulosa cells was harvested. The collapsed follicles were then opened in half using a scalpel blade and the inner follicular wall was scraped with a microbiology culture-loop (LightLabs, cat#PD104, Dallas, Texas, USA) to remove the mural layer of granulosa cells. The mural and antral granulosa cells were pooled within animal and snap frozen in liquid nitrogen and kept at -80C for later microarray analysis.

4.3.3 RNA extraction and amplification

Total RNA was extracted using Trizol extraction method according to the manufacture's instruction (Invitrogen Life Technology) and resuspended in 50µl of nuclease-free water. RNA was purified using the Arcturus *PicoPure RNA* Isolation and purification Kit (Catalog KIT0204 Applied Biosystems, Ontario, Canada) following the manufacturer's protocol. The purification process includes DNAse treatment to remove DNA and final pure RNA was recovered in 15µl of elution buffer. RNA quality was evaluated using Bioanalyzer-2100 (Agilent Technologies, Palo Alto, CA, USA) with the RNA NanoLab Chip (Catalog # 5067-1511, Agilent Technologies). Only RNA samples with RNA integrity number (RIN) greater than 5 were used for microarray hybridizations.

Five nanograms of purified RNA were used for amplification. For the superstimulation group, equal amounts of RNA from the three largest follicles were pooled and a total of 5 ng RNA from the pooled sample was used. For the control group, the same amount of RNA was obtained from the single dominant follicle. The amplification process was chosen with the intent of increasing the amount of genetic material used for microarrays. A linear amplification was performed using two 6-hour rounds of T7 RNA polymerase (RiboAmp HS^{Plus} RNA Amplification Kit; Molecular Devices, Sunnyvale, CA, USA) following manufacturer's directions. The antisense RNA (aRNA) output was measured using NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA).

4.3.4 Sample labeling, hybridization and microarray scanning

For each sample, 2.5 µg of aRNA were labeled using DY-547/647 (Red - CY5 and Green CY3) fluorescent dyes from Universal Linkage System (ULS) Labeling Kit (EA-006, Kreatech Diagnostics, Amsterdam, The Netherlands) according to the manufacturer's protocol. With the intent of removing all non-reacted ULS-labeled material, another round of purification was performed in the labeled aRNA, also using the Pico-Pure RNA Isolation Kit but without DNAse I treatment. Pure labeled aRNA was

eluted with 11 µl elution buffer. Labeling efficiency was measured using NanoDrop ND-1000. A minimum of 30 pmol/µg (dye concentration/aRNA concentration) of labeling signal was required to proceed with hybridization. A hybridization mixture was prepared using 825 ng of each cyanine (Cy3 and Cy5) labeled amplified aRNA, agilent and tomato spikes, nuclease free water, 10X blocking agent and a 25X fragmentation buffer, in a total volume of 55 µl, which was pipetted onto the hybridization slides. Three biological replicates in each group (superstimulation vs. control) were used in the experimental design, in a dye-swap set up. Overall, 6 hybridizations were performed using a custom-built bovine oligo-array slide (EmbryoGENE EMBV3 manufactured by Agilent; Design ID: 028298, GEO accession # GPL13226). The slide contained a total of 45,220 oligo-nucleotide probes. Each probe had a duplicate and the slide also included Agilent's positive and negative controls in 4x44K format. Oligo sequences were taken from the Oligo Microarray Consortium database (BOMC, http://www.bovineoligo.org).

Hybridizations were performed (Agilent Technologies Inc., Wilmington, DE, USA) using 2x GEx Hybridization Buffer HI-RPM, at 65ºC in a preheated oven for 17 hours with a rotator speed of 10 rpm. Slides were washed with two buffers from the gene expression wash buffer kit (Agilent technologies Inc. DE USA, catalogue # 5188-5327), according to manufacturer's protocol. Later, slides were dipped in 100% acetonitrile for 10 seconds at room temperature and washed with stabilization and drying solution for 30 seconds at room temperature.

The slides were scanned immediately and visualized using a Power Scanner (Tecan US Inc, Durham, North Carolina, USA). After image acquisition, scanned images were analyzed and quantified using Array-Pro Analyzer software (Media Cybernetics, Silver Spring, MD, USA).

4.3.5 Data normalization and statistical analysis

Raw signal intensity files were uploaded to the EmbryoGENE laboratory information management system and microarray analysis platform. Quality of

hybridization was evaluated using Gydle software (http://www.gydle.com/). Signal intensity data were analyzed using the FlexArray software, version 1.6.1 (Blazejczyk *et al*. 2007) The intensity of the background signal was subtracted from the median gray-scale value of the spot in question to obtain corrected signal intensity. If the background intensity was higher than the signal intensity for a spot, negative value was replaced with 0.5 as a default. Data were normalized within and between arrays using Loess and Quantile normalization methodology (GEO #: GSE45381), respectively (Bolstad *et al.* 2003). Linear Models for Microarray (Limma) data analysis was performed (implemented in FlexArray software; http://genomequebec.mcgill.ca/FlexArray/license.php) to obtain differentially expressed genes in the superstimulation group compared with the reference (unstimulated control) group (Smyth 2004, Smyth 2005), using a fold-change of ≥2 and a P-value of ≤0.05 as a threshold. To identify true positive gene changes, a false discovery rate analysis was done using the Benjamini-Hocheberg method (Benjamini & Hochberg 1995) with a fold-change of ≥2 and P-value of ≤0.05.

4.3.6 Functional annotation and pathway analysis

The list of differentially expressed gene, generated after Limma analysis, was uploaded into Ingenuity Pathways Analysis software (IPA; Ingenuity Systems, www.ingenuity.com) to identify gene networks. Gene networks were used to identify likely biological functions, molecular processes and disorders, and pathways most related to the gene list. IPA analyses are based on human and mouse studies.

4.3.7 Real-time PCR

Based on the results of microarray data, five genes (*NTS, FOS, THBS1, FN1* and *IGF2*) were selected for validation with real-time PCR. Selected genes were involved in the hypotheses being generated by the microarray analysis. Primers were designed using Primer3 v.0.4.0 website (http://frodo.wi.mit.edu/primer3/); analyzed using IDT PrimerQuest tool - Oligo Analyzer (http://scitools.idtdna.com/analyzer/Applications/OligoAnalyzer/) and BLAST analysis

was performed using NCBI database (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Selected primers were specific to the gene of interest and met the following criteria: 20 to 24 base pairs; 55 to 65^0C anneling temperature; 40 to 60% of CG content; and no hairpin, self-dimer or hetero-dimer formation. Nucleotide sequences of selected forward and reverse primers are presented in Table 1 and primers were synthesized by a commercial company (Integrated DNA Technologies (IDT) Inc., Coralville, IA, USA). Primers were tested by performing real-time PCR of cDNA from pooled granulosa cell samples. The amplicon was separated by electrophoresis on a standard 1% agarose gel to determine the size of the band. The band was cut and eluted using a QIAquick Gel Extraction kit (Cat# 28704, Qiagen, Toronto, ON, Canada), quantified using NanoDrop ND-100 and sequenced using ABI 3730*xl* DNA Analyzer. Primers were used only if the amplicon sequence matched the desired gene of interest. The amplicon was used for generating a standard curve. The range of the standard curve was 10^{-2} to 10^{-11} ng/µl. Real-time PCR was performed in a Stratagene Mx3005P fast thermal cycler (Applied Bioscience) using SYBR Green master mix (Applied Bioscience). Cycle threshold was recorded, and expression of the gene of interest was normalized to a geometric mean of three reference genes (*UBE2D2, EIF2B2* and *SF3A1*) using the Relative Expression Software Tool (REST 2009, Quiagen).

Table 4.1. Primers used for real-time PCR.

Genes	Strand	Primer sequence	Annealing Temperature (°C)
	Forward	5’-CATGAGATGGCAGTCAATTTGT-3’	53.6
EIF2B2	Reverse	5’-CTTGAACATAGGAGCACAGACG-3’	55.5
	Forward	5’-TGTGTCCCTCTTGCTGAGTTT-3’	56.4
SF3A1	Reverse	5’-ATTCCTGGTTTCACGTCTCCTA-3’	55.5
	Forward	5’-TGGACTCAGAAGTATGCGATGT-3’	55.8
UBE2D2	Reverse	5’-CTTCTCTGCTAGGAGGCAATGT-3’	56.6
	Forward	5’-AGTGTTCCCTCTTGGAAAATGA-3’	60
NTS	Reverse	5’-TCTTCCTGAATCAACTCCCAGT-3’	60.1
FOS	Forward	5’-AGGTAGAACAGTTGTCCCCAGA-3’	60

	Reverse	5’-CAAAGCCGACTTCTCATCTTCT-3’	60
	Forward	5’- TCGACTGTGAGAAGATGGAGAA -3’	59.9
THBS1	Reverse	5’- GTTGTCAAGGGTGACAAAGACA-3’	60
	Forward	5’-AGAAGAGTGAGCCTTTGATTGG-3’	59.9
FN1	Reverse	5’-AGATCATTTGTTGCCCAAGACT -3’	60
	Forward	5’- GCAACACCAGAAAAGCAAACTA -3’	59.4
IGF2	Reverse	5’-CAGATCCAAAAGGAACGAGAAG-3’	60.2

4.3.8 Radioimmunoassay

Estradiol and progesterone concentrations were measured in samples of antral fluid aspirated from superstimulated follicles and from dominant and subordinate follicles in the control group by radioimmunoassays. Slaughterhouse ovaries were used to obtain a charcoal-extracted pool of follicular fluid, which was used to prepare the standards and dilute follicular fluid samples. Standard curve ranged from 5 to 1000 pg/ml for estradiol and 0.1 to 40 ng/ml for progesterone. Samples were diluted using the charcoal-extracted pooled follicular fluid so that hormone concentrations fell within the limits of the standard curve and samples were assayed in duplicates. Estradiol was measured with a modified human double-antibody RIA Kit (Catalog # KE2D1, Coat-A-Count; Siemens Healthcare Diagnostics Inc.; Mississauga, ON, Canada), dilutions ranged from 1:25 to 1:500. Progesterone was measured using commercial radioimmunoassay kit (Catalog # TKOP1, Coat-A-Count; Siemens Healthcare Diagnostics Inc.; Mississauga, ON, Canada) and all samples were diluted 1:10. All samples for progesterone analysis were analyzed in a single assay only and the intra-assay coefficient of variation was 5.6%. Estradiol was measured in two different assays and the intra-assay coefficient of variation was 11%, while inter-assay coefficient of variation was 8.1%. Hormone data were analyzed by analysis of variance using a general linear model procedure (GLM) (SAS Learning Edition 4.1; SAS Institute; Cary, NC, USA) to compare follicular fluid from

superstimulation group vs. dominant follicle from non-superstimulated (control group) vs. subordinate follicles from non-superstimulated (control group) animals.

4.4. Results

4.4.1 Differential gene expression profile

A total of 470 genes were differentially expressed in granulosa cells from superstimulated cows compared with those of untreated control cows. Of these, 190 genes had significantly lower expression in the superstimulated group (i.e., down-regulated genes), and 280 genes had significantly higher expression in the superstimulated group (i.e., upregulated genes) compared with controls (Fig. 4.2). The 10 most up- and down-regulated genes in the superstimulation vs. control group are listed in Table 2.

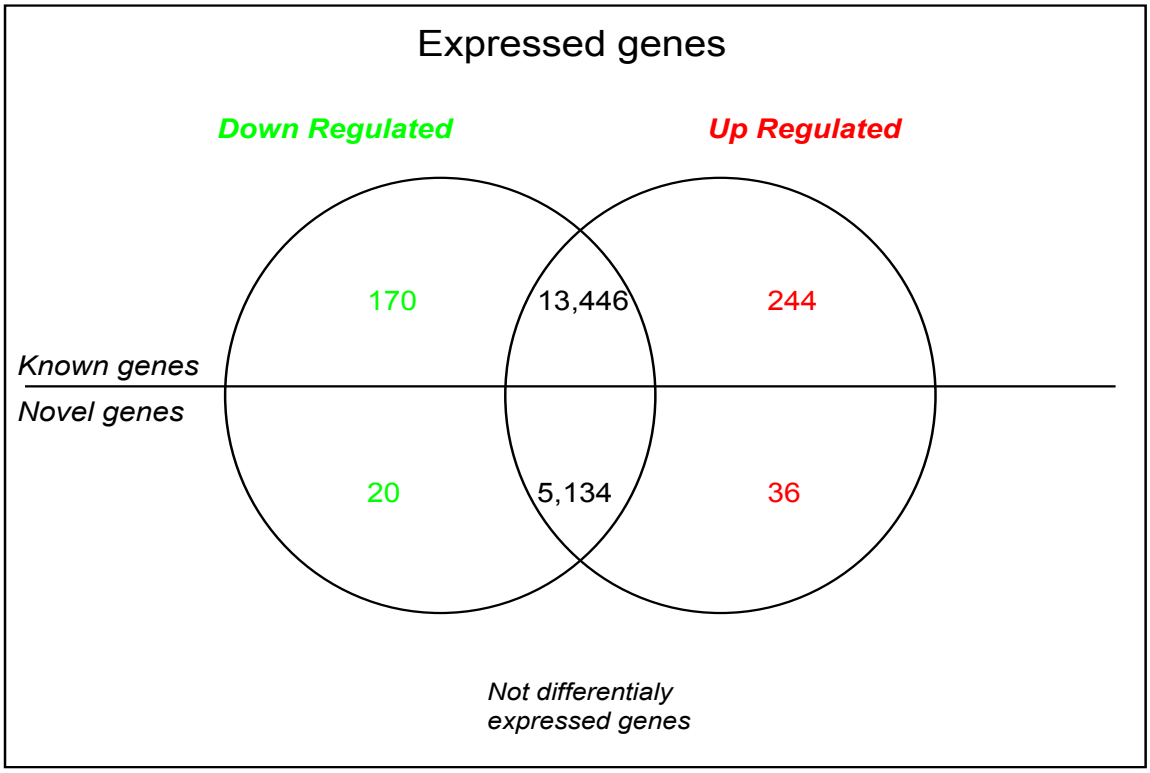


Figure 4.2. Venn diagram summarizing microarray analysis of granulosa cells of follicles from superstimulated vs untreated control cows. Detected signals were those that were ≥2 standard deviations above the mean background (129.8 ± 14.9). Linear Models for Microarray Data (Limma) was used for statistical analysis, and up- and down-regulated genes were determined using a fold-change of ≥2 and P-value of ≤0.05.

Table 4.2. Top 10 up- and down-regulated genes in granulosa cells of beef cows after ovarian superstimulation treatment compared with untreated controls (n=3 cows per group).

Genes	Description	Fold Change	P-value
Upregulated			
NTS	Neurotensin	9.987	$9.13x10^{-07}$
S100B	S100 calcium binding protein B	8.345	$8.43x10^{-03}$
IGFBP1	Insulin-like growth factor binding protein 1	7.797	$2.03x10^{-03}$
GFPT2	Glutamine-fructose-6-phosphate transaminase	7.777	$8.23x10^{-03}$

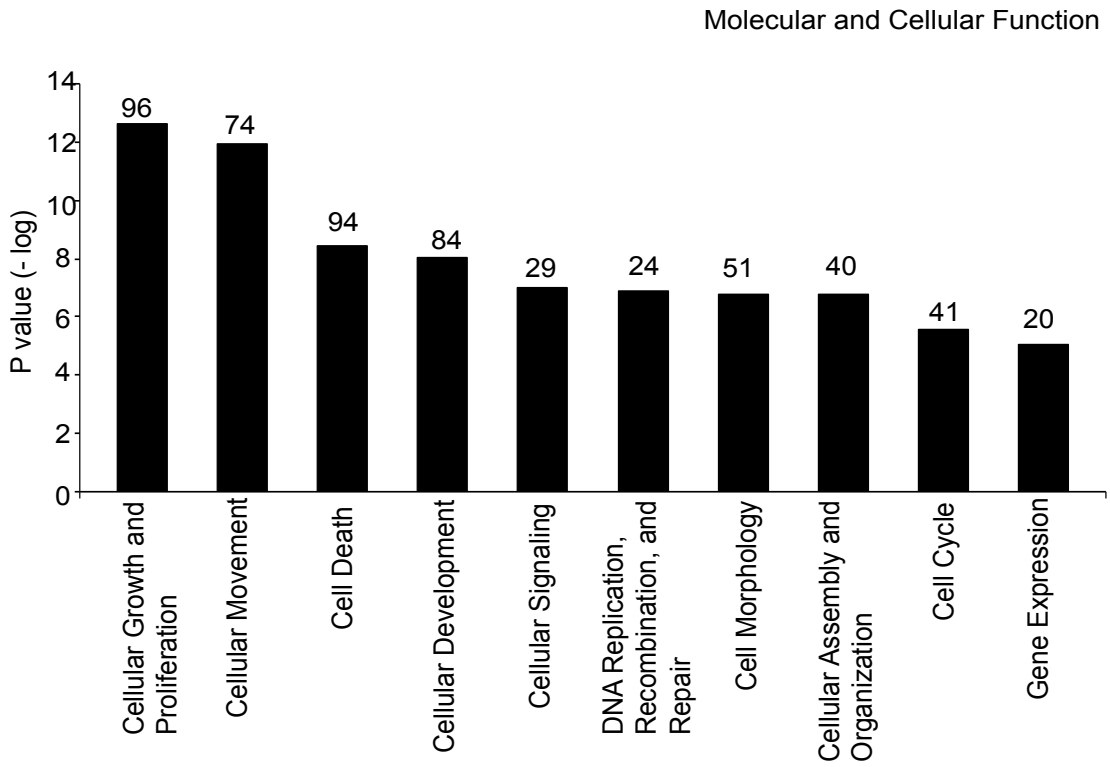
	2		
SLC39A8	Solute carrier family 39 (zinc transporter), member 8	7.775	$4.53x10^{-04}$
COBLL1	Cordon-bleu protein-like 1	7.35	$7.07x10^{-03}$
FOS	FBJ murine osteosarcoma viral oncogene homolog	6.86	$1.27x10^{-05}$
LUM	Lumican	6.006	$5.27x10^{-04}$
RCAN1	Regulator of calcineurin 1	5.892	$2.76x10^{-04}$
THBS1	Thrombospondin 1	5.685	$1.71x10^{-03}$
Down-regulated			
FES	Feline sarcoma oncogene	7.638	$1.85x10^{-03}$
NRP1	Neuropilin 1	6.482	$2.16x10^{-05}$
ANKRD43	Ankyrin repeat domain 43	5.517	$3.52x10^{-04}$
CNIH3	Cornichon homolog 3 (Drosophila)	4.944	$1.89x10^{-04}$
MGST2	Microsomal glutathione S-transferase 2	4.687	$4.36x10^{-04}$
ANGPT2	Angiopoietin 2	4.53	$3.69x10^{-02}$
LTF	Lactotransferrin	4.524	$1.35x10^{-04}$
TRIB2	Tribbles homolog 2 (Drosophila)	4.472	$8.34x10^{-03}$
SYNE1	Spectrin repeat containing, nuclear envelope 1	4.467	$7.13x10^{-03}$
MXRA8	Matrix-remodelling associated 8	4.289	$7.35x10^{-03}$

4.4.2 Function, network and pathway analyses

Cellular functions most affected by superstimulation treatment were cellular growth and development (Figure 4.3A). Regarding disease and disorders, superstimulation activated genes related to cancer, genetic disorders, and disorders of the cardiovascular system (Figure 4.3B). Network analysis identified processes involving matrix-remodeling network, more specifically genes involved in proliferation of cells, apoptosis and angiogenesis (Fig. 4.4). The most significant canonical pathways identified

by Inguinity pathway analysis were those related to *IGF* and *ERK5* signaling, oxidative stress response, and inhibition of angiogenesis (Fig. 4.5).

A)



B)

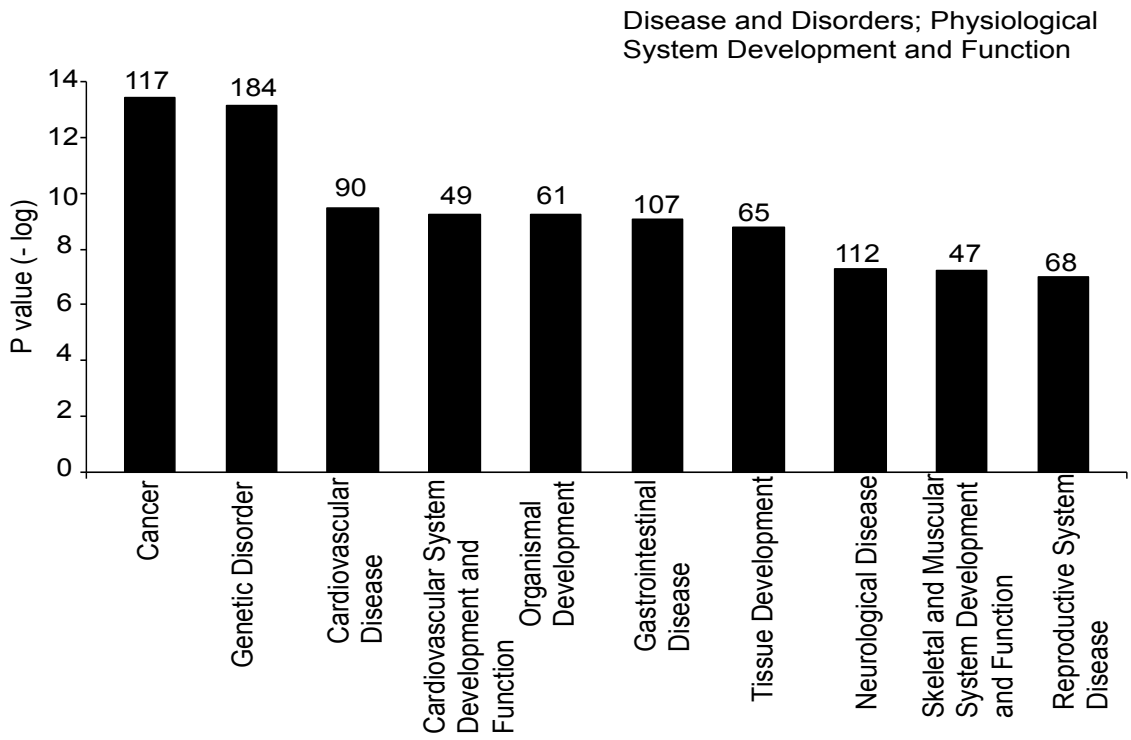


Figure 4.3. Function analysis of gene expression of granulosa cells after superstimulation treatment based on log P-value. The higher the log P-value (taller bars) the more significant the function is. Numbers on the top of the bars indicate number of genes involved with each function. Top 10 molecular and cellular functions (A) and top 10 disease and disorders/ physiological function (B) are illustrated.

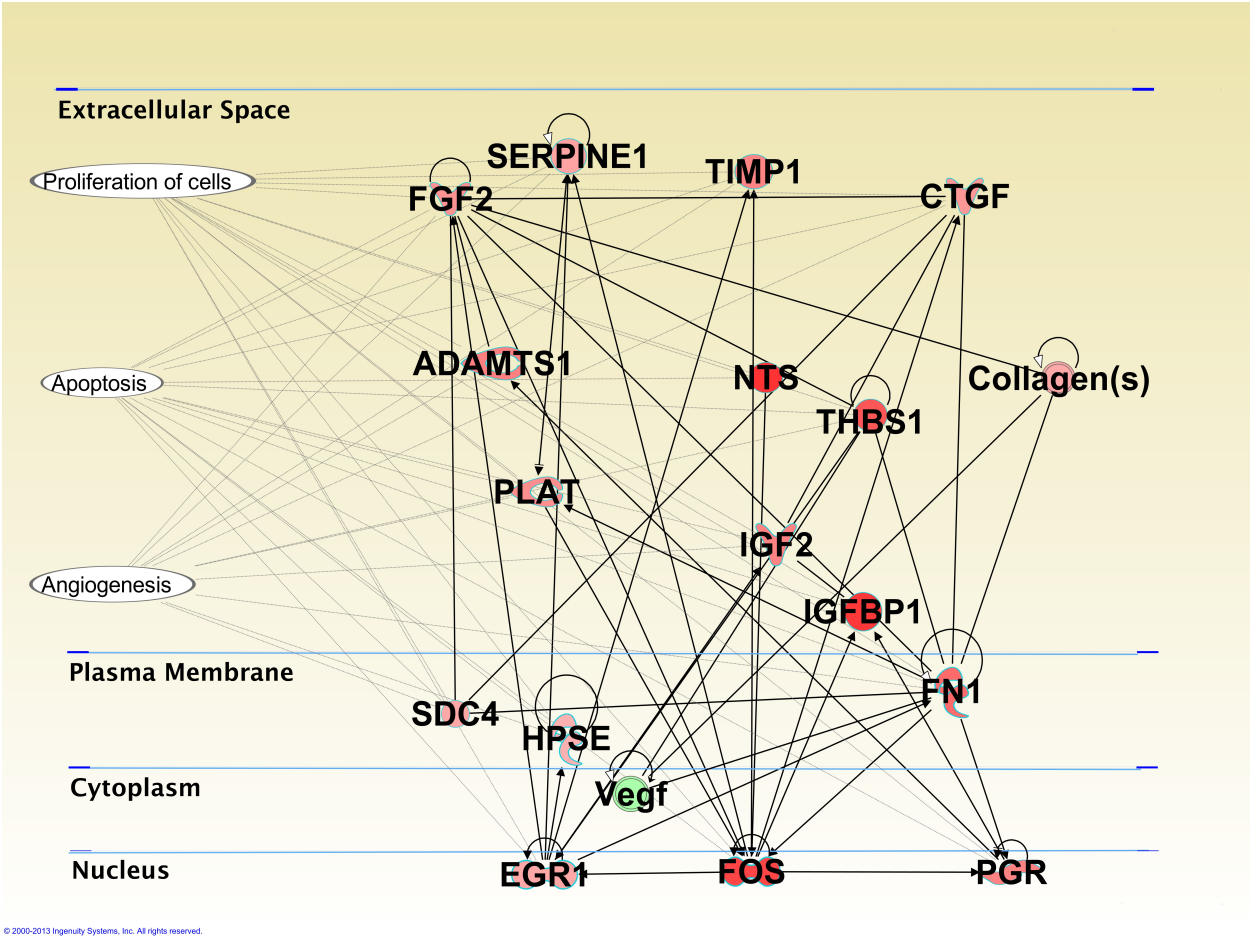
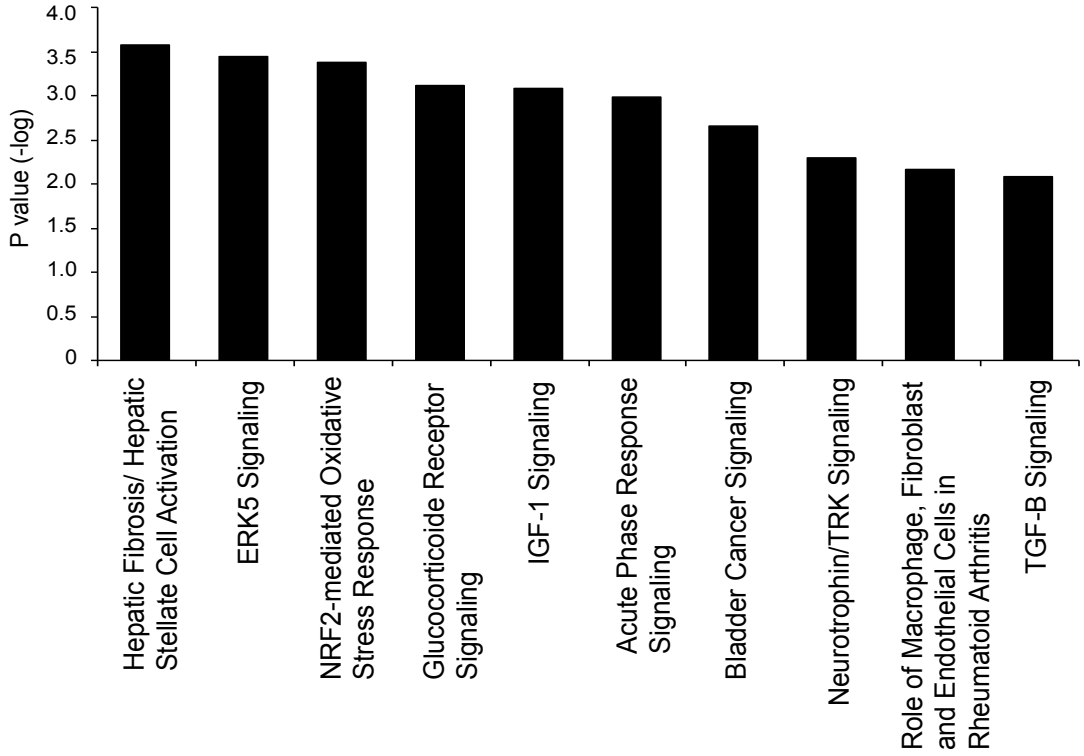


Figure 4.4. Network of genes up- or down- regulated in granulosa cells after ovarian superstimulation in cattle. All genes involved in this network are part of the matrix remodeling network; more specifically: cellular proliferation (n=16 genes), apoptosis (n=15 genes) and angiogenesis (n=11 genes). Genes are arranged into 4 horizontal compartments (nucleus, cytoplasm, plasma membrane and extracellular space) based on the subcellular location of their gene products. The differences in color intensity of molecules show the degree of up- (red) or down- (green) regulation and connecting lines indicate a known relationship between molecules. The genes found to be upregulated were: EGR1, early growth response 1; FOS, FBJ murine osteosarcoma viral oncogene homolog; PGR, progesterone receptor; SDC4, syndecan 4; HPSE, heparanase; FN1, fibronectin 1; IGFBP1, insulin growth factor binding protein 1; IGF2, insulin growth factor 2; PLAT, plasminogen activator tissue; ADAMTS1, ADAM

metallopeptidase 1; NTS, neurotensin; THBS1, thrombospondin 1; Collagens; FGF2, fibroblast growth factor 2; SERPINE1, serpin peptidase inhibitor class E member 1; TIMP1, metallopeptidase inhibitor 1; CTGF, connective tissue growth factor. The only down-regulated gene was VEGF, vascular endothelial growth factor. Included genes belonged to transcription regulators (EGFR, FOS), ligand-dependent nuclear receptors (PGR), cytokine or growth factors (FGF2, CTGF, IGF2), peptidases (ADAMTS1, PLAT), enzymes (HPSE, FN1) or other complex group (VEGF, SERPINE, SDC4, TIMP1, NTS, THBS1, Collagens, IGFBP1) categories.

A)



B)

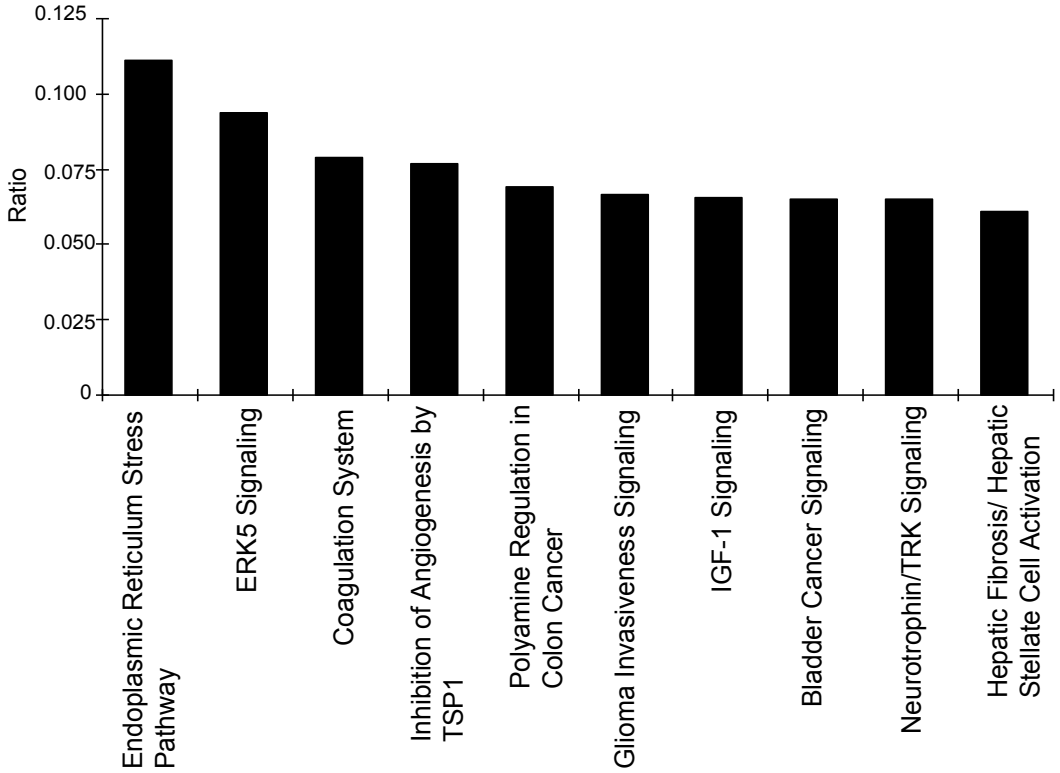


Figure 4.5. Canonical pathways analysis of gene expression of granulosa cells after superstimulation treatment using Ingenuity Pathway Analysis software (IPA). A) Top 10 pathways analysis based on $-\log_{10}$ P-value. B) Top 10 pathway analysis based on a score ratio. The score ratio was calculated by IPA and is the number of differentially expressed molecules in the gene list/ number of genes known to be involved in the pathway.

4.4.3 Real-time PCR validation

Based on microarray data and function analysis, 5 genes were selected for validation with real-time PCR (i.e., *NTS, FOS, FN1, THBS1* and *IGF2*). After quantification in three independent biological replicates from superstimulation and control groups, differential expression was validated for 4 of the 5 genes (90% confidence level; $P \leq 0.1$; Fig. 4.6).

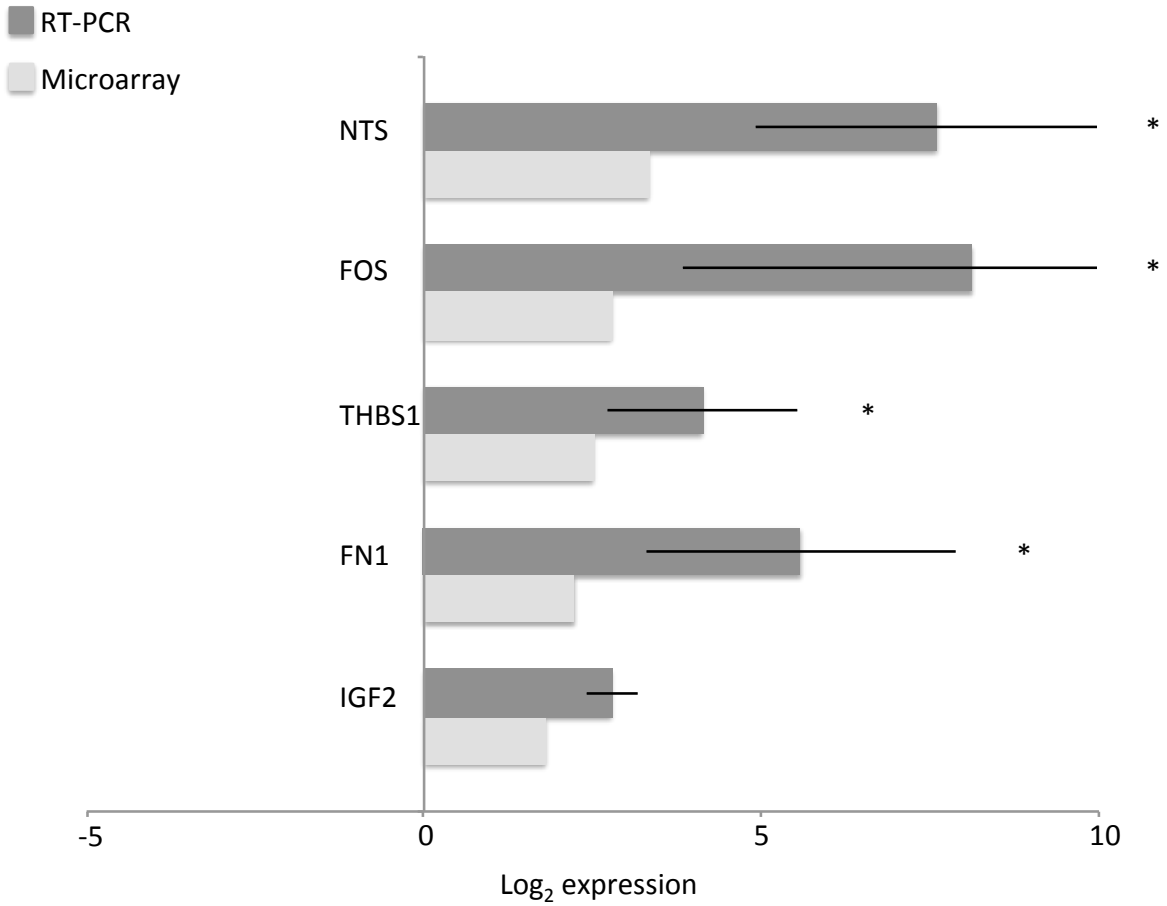


Figure 4.6. Quantification (log_2 of fold-change; mean ±SEM) of the mRNA profile of granulosa cells from cows after superstimulation treatment compared with negative control (no superstimulation) using real-time PCR (n=3 cows per group). Light grey bars represent the differential level of expression of transcripts detected in the microarray experiment, while dark grey bars represent the differential level of expression of the same transcripts obtained by real-time PCR. *Values are greater in the superstimulation group than in the untreated control group (P≤0.1).

4.4.4 Hormone levels in follicular fluid

Follicular fluid from superstimulated follicles and from dominant follicles of the control group had a higher estradiol levels compared with subordinate follicles of the control group (153.8 ± 32.7, 160.4 ± 64.9, 0.05 ± 0.02 ng/ml; mean±SEM, respectively;

P=0.01). Follicular fluid progesterone levels did not differ among groups (99.8 ± 19.7, 74.0 ± 18.5, 108.6 ± 62.7,P=0.8 ng/ml; mean±SEM, respectively; P=0.8). Estradiol:progesterone ratio was greater in follicular fluid from superstimulated follicles and from dominant follicles of the control group compared with subordinate follicles of the control group (3.5 ± 0.8, 2.2± 1.2, 0.004 ± 0.002; mean±SEM, respectively; P=0.03); however, the ratio did not differ between the superstimulated follicles and the dominant follicle.

4.5 Discussion

Ovarian superstimulation protocols are used widely in monovular species as a component of assisted reproductive technologies. Superstimulation involves the use of follicle stimulation hormone (FSH) to stimulate continued growth of a follicular cohort to a pre-ovulatory stage. The effect of superstimulation on follicular dynamics in cattle is well known (Adams *et al.* 1992b, Nasser *et al.* 1993, Adams 1998, Mapletoft *et al.* 2002); however, the genomic response of bovine follicles to gonadotropin stimulation has not been established. Results of the present study document that molecular pathways related to granulosa cell differentiation are altered in superstimulated follicles compared to dominant follicles of unstimulated follicular waves at equivalent stage of development (that is, identical durations from wave emergence to progesterone withdrawal and exogenous LH treatment, and identical post-LH period). Results provide rationale for three hypotheses: Compared to the granulosa cells of single (natural) dominant follicles, superstimulation treatment 1) results in granulosa cells that lag behind in maturation and differentiation (most of the upregulated genes are markers of the follicular growing stage), 2) activates genes involved with the oxidative stress response, and 3) disturbs angiogenesis.

Compared to a single dominant follicle of a naturally-occurring follicular wave, ovarian superstimulatory treatment delays selection and results in development of

multiple dominant follicles (Adams et al., 1993b). However, results of the present study suggest that the multiple pre-ovulatory follicles that develop after superstimulation are not typical dominant follicles. The list of upregulated genes in the superstimulation group in the present study is similar to those of other studies (Gilbert *et al.* 2011, Gilbert *et al.* 2012) from dominant follicles before the LH surge at Day 14 of estrous cycle. It is important to note that the granulosa cells from our superstimulation group were collected 24 hours after exogenous LH surge (i.e., close to the expected time of ovulation). The principal molecular functions altered by ovarian superstimulation were those associated with cell growth and proliferation, providing rationale for the hypothesis that superstimulation delays granulosa cell differentiation. That is, granulosa layer of single preovulatory follicles at the time of collection have differentiated beyond the growing stage by downregulating genes associated with cell growth and proliferation and/or perhaps responding better to LH signaling compared to the granulosa cells from superstimulated follicles. It would be interesting to determine if delaying the LH surge after superstimulation treatment would result in follicular maturation and a better fertility outcome. It is worth noting that not all functions are equally affected, e.g., steroidogenic function (intrafollicular estradiol concentration, E2:P4 ratio) was very similar between the dominant and superstimulated follicles.

The extracellular matrix plays a prominent role in ovarian function by participating in processes such as cell migration, proliferation, growth, and development of follicles (Berkholtz *et al.* 2006a, Berkholtz *et al.* 2006b). In the present study, upregulated genes involved with remodeling of the extracellular matrix included *NTS, FOS, THBS1, FN1, ADAMTS1, CTGF*, genes from the *IGF* family, and those involved in collagen formation. A specific network was built to show how extracellular matrix remodeling genes interact with each other, and how superstimulation affected this process (Figure 4.4).

The *NTS* gene encodes a tridecapeptide, neurotensin, found in the hypothalamus. Neurotensin is known to mediate the positive feedback of estradiol in the GnRH neurons responsible for inducing the preovulatory LH surge (Smith & Jennes 2001). Neurotensin

was thought to be localized exclusively in the nervous system, but results of recent studies document the presence of *NTS* mRNA in the ovaries (Hernandez-Gonzalez *et al.* 2006, Kerr *et al.* 2009, Gilbert *et al.* 2011). Little is known about the role of neurotensin in ovarian cells, but expression of *NTS* mRNA in granulosa and cumulus cells is high before the LH surge and decreases thereafter as ovulation approaches (Hernandez-Gonzalez *et al.* 2006, Gilbert *et al.* 2011). Based on the rt-PCR analysis, *NTS* was upregulated 7.6 folds (10-fold in microarrays data) after ovarian superstimulation suggesting that treatment is associated with delay in cell differentiation and/or delayed follicular response to the LH surge. *FOS* (6.9-fold in microarray data and 8.1-fold in rtPCR) and *FOSL1* (2.1-fold in microarrays) transcriptions was also upregulated, and both activate NTS synthesis (Evers *et al.* 1995). Expression of *FOS* has been associated with cell proliferation and development (Delidow *et al.* 1990) and FSH rapidly increases *FOS* expression in immature rat granulosa cells (Delidow *et al.* 1992). Expression of *FOS* decreases with luteinization (Rusovici & LaVoie 2003) and is already low in bovine granulosa cells by 6 hours after the LH surge (Gilbert *et al.* 2011). Therefore, we conclude that granulosa cells of superstimulated follicles were either unable of shut down *NTS, FOS* and *FOSL1* transcription or at least were slower to respond to exogenous LH.

Connective tissue growth factor (*CTGF*) has also been implicated in tissue remodeling. *CTGF* mRNA is expressed abundantly in granulosa cells of pre-antral and early antral follicles in rats (Harlow *et al.* 2002) and pigs (Wandji *et al.* 2000), but its expression is down-regulated in pre-ovulatory follicles in both species. Expression of *CTGF* is influenced by local estrogen, which in turn, is modulated by the effect of FSH on granulosa cells (Harlow *et al.* 2007). Perhaps expression of *CTGF* will be a useful marker for granulosa cell maturity since many studies have reported an inverse relationship between *CTGF* expression and granulosa cell differentiation (Wandji *et al.* 2000, Harlow *et al.* 2002, Harlow & Hillier 2002, Liu *et al.* 2002, Schindler *et al.* 2010). Although the follicular fluid concentrations of estradiol were similar between the two groups in the present study, *CTGF* was upregulated in the granulosa cells of superstimulated follicles, again suggesting a delay in follicular maturation. Further, expression of three other genes involved in matrix remodeling, *SERPINE*, fibronectin 1

(*FN1*) and I*GF2* was also elevated in the superstimulation group. *SERPINE* is down-regulated in bovine granulosa cells after LH treatment (Gilbert *et al.* 2012) and *FN1* expression is reported to be inversely related to follicle maturation (Colman-Lerner *et al.* 1999, Yasuda *et al.* 2005, Berkholtz *et al.* 2006b). Insulin-like growth factor proteins are produced by the granulosa cells and have been shown to have a synergetic effect with FSH to induce cellular growth and proliferation (Hammond *et al.* 1985). The expression of *IGF2* is increased in follicles collected at day 5 (D0=ovulation) and decreased in follicles at day 8 (de la Sota *et al.* 1996). The increased expression of the genes described above reflect that matrix remodeling is active, probably due to follicular grow.

Matrix remodeling is not only important in follicle growth and development, but also during ovulation and CL formation (Berkholtz *et al.* 2006a). In the present study, some upregulated genes in the matrix-remodeling network (*HPSE, EGR1, TIMP1, PLAT*) are markers of the initiation of ovulatory process (Gilbert *et al.* 2011, Gilbert *et al.* 2012). *HPSE* encodes heparanase that cleaves heparan sulfate (one of the tissue glycosaminoglycans) during matrix remodeling and is highly expressed in bovine granulosa cells 12 hours after GnRH treatment. Heparanase was suggested to be a novel member of the LH-induced ECM-degrading enzyme family involved with follicular rupture (Klipper *et al.* 2009). Likewise, plasminogen activator (*PLAT*) creates functionally redundant mechanisms for plasmin formation during ovulation (Sayasith *et al.* 2006) and is highly expressed in granulosa cells of pre-ovulatory follicles in rats (Galway *et al.* 1990, Leonardsson *et al.* 1995). The expression of tissue inhibitor metalloproteinase 1 (*TIMP1*) is increased up to 4 h after hCG treatment in granulosa cells of immature PMSG-primed rat ovaries and gradually decreases afterwards (Li & Curry 2009). It is interesting to note that *HPSE, PLAT* and *TIMP1* expression was higher in superstimulated follicles. In contrast, *EGR1* expression is expected to decrease near the time of ovulation (Sayasith *et al.* 2006), but failed to do so in superstimulated follicles. Over-expression of early growth response 1 (*EGR1*) stimulated the expression of many genes in the prostaglandin biosynthesis pathway and increased expression of LH receptor mRNA (Sayasith *et al.* 2006). Its expression in superstimulated follicles from women was associated with increased oocyte competence (Hamel *et al.* 2008). It is apparent from our

results that expression of some but not all genes involved in the ovulatory cascade are affected by superstimulation. In the present study, LH was given 24 hours after the end of superstimulation treatment. Perhaps the exogenous LH is driving follicles that are not fully matured to reach the pre-ovulatory molecular stage and ovulate. However, this hypothesis needs to be further investigated.

In the present study, genes related with the stress response (Fig. 4.5) were activated in granulosa cells from superstimulated follicles. Our results are supported by similar findings in embryos from superstimulated donors (Mundim *et al.* 2009), where genes related to oxidative stress response tended to be activated. In the present study, the *NRF2 oxidative stress response* pathway was one of the most activated pathways in the superstimulation group. *NRF2* interacts with other transcription factors within the nucleus, such as *CREB, ATF4* and *FOS*, to activate antioxidant response elements (Gudi *et al.* 2000) to balance the oxidation level of the intracellular environment (Huang *et al.* 2000). The *endoplasmic reticulum (ER) stress pathway* was also activated in granulosa cells of superstimulated follicles in the present study. Many diseases are associated with cellular stress responses, one of which is cancer (Cerutti 1989), which was identified in the top disease and disorders function list. Perhaps, the oxidative stress response comes from too many follicles to support by the ovarian vasculature. However, this hypothesis should be further tested.

Some of the upregulated genes in this study such as *FOS, FN1, EGR1, SDC4, THBS1* are demonstrated to induce the expression of pro-apoptotic proteins (Delidow *et al.* 1990, Sakata *et al.* 2000, Yasuda *et al.* 2005, Hou *et al.* 2008, Garside *et al.* 2010a). *THBS1* encodes a glycoprotein, thrombospondin,1 that is a component of the extra-cellular matrix. Thrombospondin mainly acts as an anti-angiogenic factor; however it also promotes atresia of rat granulosa cells in vitro (Garside *et al.* 2010a, Garside *et al.* 2010b). *THBS1* is highly expressed in small follicles from bovine ovaries (Greenaway *et al.* 2005) as well as in small and atretic follicles of many other species (Thomas *et al.* 2008, Garside *et al.* 2010b, Zalman *et al.* 2012). *IGF1* inhibits *THBS1* transcription in cultured rat granulosa cells and its expression is further lowered when FSH is added in

culture (Dreyfus *et al.* 1992, McGray *et al.* 2011). In contrast, thrombospondin 1 is highly expressed in bovine granulosa cells when FSH is added in culture but LH had no effect (Greenaway *et al.* 2005). *THBS1* expression was upregulated 4.1 fold (rt-PCR and 5.7 fold in microarrays) in superstimulated follicles. Perhaps, expression of this group of genes indicates that even though superstimulation rescued follicles from atresia, some molecules involved with that pathway are still being triggered. In contrast, other known anti- atresia markers (*ADAMTS1* and *TIMP*) were also upregulated after superstimulation. Protein encoded by *ADAMTS*1 are known to prevent atresia as mice with *ADAMTS1* null ovaries had many unusual atretic follicles (Shozu *et al.* 2005), demonstrating the importance of this gene in granulosa cell health. Proteins from the tissue inhibitor of metalloproteinase gene family (TIMP) are also known to be stimulated by FSH and to prevent atresia (Goldman *et al.* 1997). Therefore, superstimulation prevent atresia by increasing expression of some, but not all, anti-apoptotic genes.

Superstimulation treatment in the present study influenced genes involved in angiogenesis; i.e., *VEGF*, angiopoietin 2, and *THBS1*. Expression of angiogenic factors have been reported to be stage dependent in granulosa cells, increasing during pre-ovulatory stage (Christenson & Stouffer 1997, Laitinen *et al.* 1997, Hazzard *et al.* 1999, Schams *et al.* 2001, Reisinger *et al.* 2007, Shimizu *et al.* 2007, Berisha *et al.* 2008), but the molecular mechanisms involved are not fully known. Angiopoietin 2 destabilizes vascular structures and induces vascular remodeling (Yancopoulos *et al.* 2000). *THBS1* also encodes an anti-angiogenic protein (Greenaway *et al.* 2005). *THBS1* inhibits *VEGF* levels in the ovary directly via the low-density lipoprotein receptor-related protein-1 (*LRP-1*) (Greenaway *et al.* 2007). Inhibition in expression of angiogenic factor (*VEGF*) and increased expression of anti-angiogenic factor (*THBS1*) was consistent with the results of pathway and function analysis wherein cardiovascular system development was identified as one of the top disease and disorder functions affected. Results suggest that angiogenesis may be inhibited, and perhaps corpus luteum formation may be disturbed after superstimulation treatment.

In conclusion, ovarian superstimulation activates genes involved with the *NRF2* oxidative stress response and endoplasmic reticulum stress response, apoptosis, disturbance of angiogenesis and matrix remodeling. Moreover, ovarian superstimulation was associated with up-regulation of growth-related genes in follicular granulosa cells. Results are consistent with the hypothesis that, follicles that undergo superstimulation lag behind in maturational development and response to LH.

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CHAPTER 5

TRANSCRIPTOME ANALYSIS OF GRANULOSA CELLS AFTER A SHORT vs. LONG FSH-STIMULATED FOLLICULAR GROWING PHASE IN CATTLE

Dias F.C.F., Khan M.I., Sirard M.A., Adams G. P., Singh J.

Relationship of this study to the dissertation

The length of growth of the ovulatory wave may result in a more mature or perhaps aged oocyte. The first study of this thesis (Chapter 3), we examined the effect of three different superstimulation protocols (a short, a long, and a long with continuous FSH support) on oocyte competence after *in vitro* fertilization. In Chapters 4, 5 (the present one) and 6, we analyzed the gene expression of granulosa cells after similar superstimulation protocols (a short, a long, and a long with continuous FSH support). In the present chapter, we used microarray analysis to compare gene expression in granulosa cells after short (4-day) superstimulation versus long (7-day) superstimulation protocols.

5.1 Abstract

In this study, a genome-wide bovine oligo-microarray was used to compare the gene expression of granulosa cells collected from bovine follicles after a short vs. long growing phase induced by exogenous FSH. Cows were allocated randomly to two groups (Short FSH and Long FSH, n=6 cows per group). Granulosa cell samples from three cows were used for microarray analysis and from the remaining three cows for qPCR validation. A new follicular wave was induced using ablation of follicles ≥5 mm in diameter by transvaginal ultrasound-guided needle aspiration, and a progesterone-releasing device (CIDR) was placed in vagina. The Short FSH group was given eight injections of 25 mg FSH at 12-h intervals starting on the day of wave emergence (Day 0), whereas Long FSH group was given fourteen injections of 25 mg FSH treatment. Both groups were given prostaglandin $F_{2\alpha}$ im twice, 12 h apart, but on Day 3 for the Short FSH group and on Day 6 for the Long FSH group. CIDR devices were removed at the time of second prostaglandin injection, 25 mg pLH was given im 24 h after CIDR removal, and cows were ovariectomized 24 h later. Granulosa cells were collected for RNA extraction, amplification and microarray hybridization. To translate microarray results into a physiological context, a list of differentially expressed transcripts were biologically annotated. Of 1031 differentially expressed genes, 416 were down-regulated and 615 genes were upregulated. To validate the microarray results, 7 genes were selected for Real Time-PCR (NTS, PTGS2, PTX3, RGS2, INHBA, CCND2 and LRP8). Four genes had a tendency to differ ($P<0.1$) between the two groups; however, Real Time-PCR results for all genes followed same trend as that indicated by the microarrays. Most of the upregulated genes in the Long FSH group are markers of LH responsiveness and proximity to ovulation, suggesting that these follicles were better able to respond to the LH surge than the short FSH group. Results provide rationale for the hypothesis that an extended period of FSH stimulation induces greater maturity and LH responsiveness of follicles than conventional short FSH stimulation.

Keywords : cattle, follicular waves, follicle growth, granulosa cells, gene expression, genomic analysis, microarrays, ovary, superstimulation.

5.2 Introduction

A surge in peripheral FSH concentrations consistently precedes emergence of a new follicular wave in cattle (Adams *et al.* 1992b). A follicular wave is characterized by the emergence of a growing cohort of FSH dependent follicles one of which is selected for continued growth (dominant follicle) while remaining subordinates follicles regress (Ginther *et al.* 1989a). The estrous cycle in cattle is composed of 2 or 3 follicular waves; the ovulatory wave is the last wave of the cycle and culminates in the ovulation of the dominant follicle. The rise and fall in circulating FSH associated with wave emergence and selection of the dominant follicle encompasses a period of 4-5 days, and is similar among waves (Jaiswal et al., 2004). The growing phase of the ovulatory wave is 9 days in 2-wave cycles and 6 days in 3-wave cycles (Ginther *et al.* 1989c). In a 2-wave cycle, the first 6 days of the growing phase of the ovulatory follicle is under a high-progesterone environment, while only the first 3 days of that of a 3-wave cycle is under a high progesterone environment (Jaiswal *et al.* 2009). Thereafter, progesterone concentrations are low (for both 2- and 3-wave cycles) for approximately 3 days following luteolysis (Jaiswal *et al.* 2009).

Follicles maintained under a low-progesterone environment for extended periods result in over-dominant persistent follicles (Adams et al., 1992) with decreased oocyte competence (Lee *et al.* 1988, Savio *et al.* 1993, Mihm *et al.* 1994, Noble *et al.* 2000). However, the effect on fertility of differing progesterone exposure during follicular growth (analogous to 2- or 3-waves cycle) is contradictory (Knopf *et al.* 1989, Ahmad *et al.* 1997, Townson *et al.* 2002, Bleach *et al.* 2004) Jaiswal et al., 2009). Recently, we reported that extending progesterone exposure during the growing and early-static phase of the ovulatory follicle by 3 days (analogous to an 2-wave estrous cycle) was not associated with an adverse effect on oocyte competence of a single dominant follicle (Dias *et al.* 2012b) or multiple dominant follicles after superstimulation (Dias *et al.* 2012a). Therefore, these results refute the notion that oocytes from 2-wave cycles are less

fertile than that of 3-wave cycles. Moreover, extending the growing phase by 3 days under progesterone exposure during superstimulation (analogous to a 2-wave cycle) resulted in collection of more mature oocytes (Dadarwal 2012), and a better ovarian superovulatory response (Dias *et al.* 2012a, Garcia Guerra *et al.* 2012). The effect of extending the growing phase in a superstimulation under a high progesterone environment (similar to 2-wave cycles) on the genomic expression of follicular cells and the oocyte is unknown.

During folliculogenesis, somatic cells and germ cells interact through complex paracrine signaling. The presence of granulosa cells is crucial for oocyte meiotic maturation (Carabatsos *et al.* 2000), and increase the efficiency of energy uptake by the oocyte (Eppig 1976, Haghighat & Van Winkle 1990). The paracrine effect of granulosa cells on the oocyte extends the growth, development (Thomas & Vanderhyden 2006) and transcriptomic activity of the oocyte (De La Fuente & Eppig 2001). It is therefore evident that granulosa cell analysis is a representative model of assessing follicular and oocyte health status. Granulosa cells from superstimulated follicles are routinely used as a control in many research projects. However, the questions remain about whether multiple dominant follicles from superstimulation are comparable to normal single dominant follicles, and what superstimulation protocol will lead to granulosa cell function closest to that of natural cycles. Our research group conducted a recent study (Chapter 3) that evaluated the molecular status of granulosa cells after superstimulation and concluded that follicles that undergo a standard 4 days superstimulation protocol are behind in their differentiation status compared to dominant follicle from a natural cycle. Therefore it will be interested to see if extending superstimulation protocol will perhaps allow follicles to reach the right stage of development and maturity.

The objective of this study was to compare the transcriptomic profile of granulosa cells from ovarian follicles exposed to a short (conventional 4 day treatment) or long (7 day treatment) duration of the FSH-stimulated growing phase (analogous to 3- or 2-wave cycle, respectively). Our hypothesis is that extending the superstimulation protocol by 3 days will allow granulosa cells to mature and respond better to exogenous LH treatment.

Therefore, we expect markers of maturity, cellular health and survival and also markers of post LH surge to be upregulated after 7-day FSH stimulation protocol.

5.3 Material and Methods

5.3.1 Animals and Treatments

Twelve cross-bred beef cows weighing 515 to 795 kg, and maintained in outdoor pens, were used. All procedures were conducted in accordance with the guidelines of the Canadian Council in Animal Care and were approved by University of Saskatchewan Protocol Review Committee.

Experimental protocol (Figure 5.1) was designed to perform 4-day or 7-day FSH treatment starting at the time of follicular wave emergence and during the functional luteal phase to obtain granulosa cells 24 hr after LH treatment. To synchronize estrus, a two i/m treatments with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; 500 μg of cloprostenol; Estrumate®, Schering-Plough Animal Health, Pointe-Claire, PQ, Canada) at 14-day intervals were performed. Daily ultrasonography examination was performed after second $PGF_{2\alpha}$ injection until ovulation was detected. Five to 8 days after ovulation, ablation of all follicles ≥5 mm in diameter was performed using transvaginal ultrasound-guided ablation method with the intent to synchronize the emergence of a new follicular wave 1 day later (Bergfelt DR *et al.* 1994). An intravaginal progesterone-releasing device, CIDR-B (Pfizer Canada Inc., QC, Canada) was placed in the vagina immediately after follicle ablation.

The cows were allocated randomly to two groups (Figure 5.1): a) Short FSH group (n=6); and b) Long FSH group (n=6). Starting 1 day after follicle ablation, i.e. in the day of wave emergence (Day 0), cows in Short FSH group were given 8 im treatments of FSH (Folltropin-V; Bioniche Animal Health, Belleville ON, Canada; each equivalent to 25 mg of NIH-FSH-P1) at 12-hour intervals over 4 days, whereas cows in the Long FSH group were given 14 im treatments of FSH over 7 d (each dose equivalent to 25 mg of NIH-FSH-P1). Cows in the Short FSH group were given two luteolytic im treatments of $PGF_{2\alpha}$ 12 h apart in Day 3, whereas the cows in the Long FSH group were given this treatment in Day 6. In all animals, the CIDR was removed concurrent with the

second prostaglandin treatment. Cows were given 25 mg pLH im (Lutropin-V, Bioniche Animal Health) 24 hours after CIDR removal. Animals were ovariectomized 24 hours after pLH treatment and ovaries were brought to the laboratory for further samples collection.

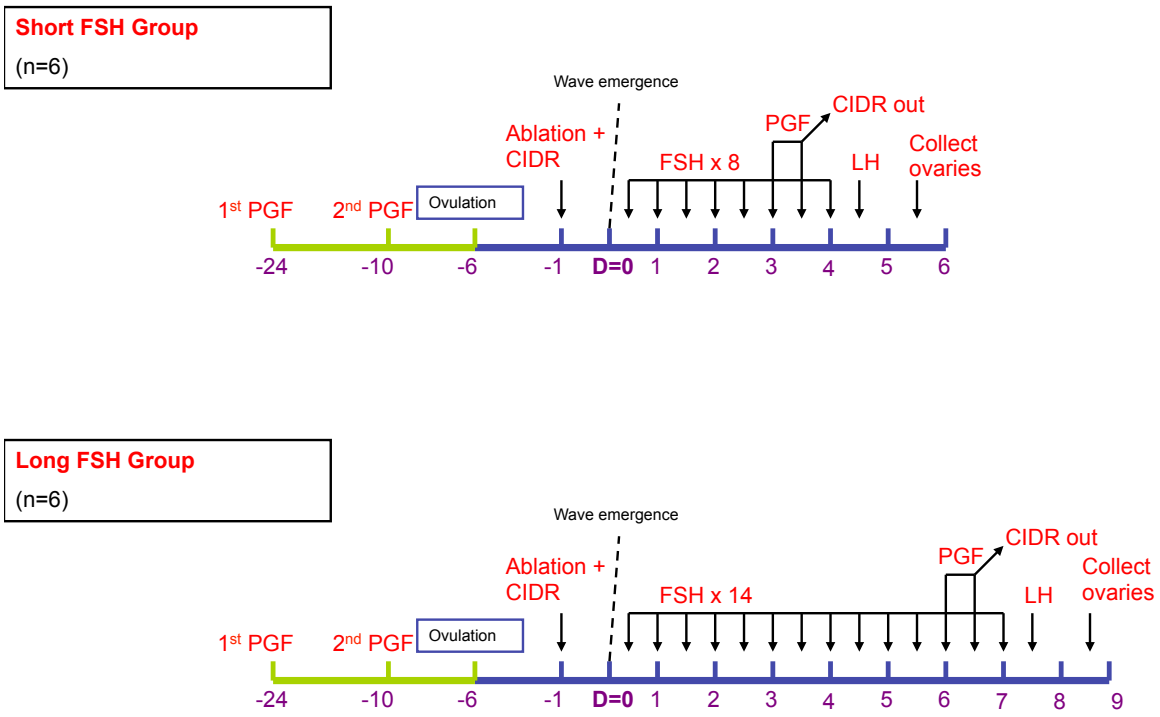


Figure 5.1 Experimental protocol used to test the effect of duration of the growing phase of ovulatory follicles on gene expression in granulosa cells. Five to 8 days after ovulation, follicles ≥5 mm were ablated and a CIDR was placed intravaginally. FSH treatment started at wave emergence (Day 0). Cows in the Short FSH group were given 8 injections of FSH at 12-hour intervals over 4 days, whereas cows in the Long FSH group were given 14 doses of FSH over 7 d. Two im treatments with $PGF_{2\alpha}$ were given on Day 3 in Short group and Day 6 in the Long FSH group. The CIDR was removed at the time of second $PGF_{2\alpha}$ injection, LH was given 24 hours after CIDR remova, and cows were ovariectomized 24 hours after LH treatment.

5.3.2 Tissue Collection

Ovariectomies were performed using a colpotomy approach modified slightly from that previously described (Singh *et al.* 1998). Briefly, caudal epidural anesthesia was induced Lidocaine HCL 2% and Epinephrine USP (5ml; Bimeda-MTC Animal Health Inc., Lavaltrie, QC, Canada) at the sacro-coccygeal or first intercoccygeal space. The perineum was scrubbed using an iodine-based detergent and solution. A small incision in the cranial aspect of the vaginal fornix was made using a scalpel blade and the peritoneum was manually ruptured to allow access to the peritoneal cavity. Local anesthesia of ovarian pedicle was induced using a lidocaine-soaked gauze, and a plastic clip was placed in the mesovarium to compress the ovarian vessels. The chain of an ecraseur (19" Chassaignac; German-made; Jorgensen Lab, Colorado, USA) was looped around the ovary and slowly tightened until the ovary was excised. The ovaries were placed in polyethylene bags, kept in ice, and transported to the laboratory within 5 minutes after collection. The number of follicles was counted and the three largest follicles were selected. The selections were based on previous ultrasound-based sketches? and confirmed using a ruler to measure follicle diameter once the follicles were opened. The goal was to collect antral and mural granulosa cells. Antral granulosa cells are the cells that are easily found free in the follicular fluid, while the mural granulosa cells surround the inner wall of the follicle. To collect the antral granulosa cells, selected follicles were aspirated using a 20 gauge needle and a syringe. Each follicle was flushed 3 times with Dulbecco's phosphate buffer saline (dPBS, Invitrogen Corporation, catalog 14190-144, Burlington, ON, Canada). The cumulus-oocyte-complex (COC) was then searched for and separated from follicular aspirates. Undiluted follicular aspirates were centrifuged to separate the follicular fluid from antral granulosa cells (pellet). The now-collapsed follicles were sliced in half using a scalpel blade and measured. The inside of the follicular wall was scraped with a microbiology culture-loop (LightLabs, cat#PD104, Dallas, USA) to remove the mural granulosa cells, which were combined with the antral granulosa cells. So the complete granulosa sample (mural and antral) were then snap-frozen in liquid nitrogen and kept at -80^{o}C for microarray and RT-PCR analyses. .

5.3.3 RNA extraction and amplification

Total RNA was extracted using Trizol extraction method according to the manufacture's instruction (Invitrogen Life Technology) and resuspended in 50µl of nuclease-free water. RNA was purified using the Arcturus® *PicoPure® RNA* Isolation and purification Kit (Catalog KIT0204 Applied Biosystem® Ontario, Canada) following manufacture's protocol. The purification process includes DNAse treatment to remove genomic DNA and final purified RNA was recovered in 15µl of elution buffer. RNA quality was evaluated using Bioanalyzer-2100 (Agilent Technologies, Palo Alto, CA, USA) with the RNA NanoLab Chip (Catalog # 5067-1511, Agilent Technologies). RNA samples with RNA integrity number (RIN) greater than 5 were used for microarray hybridizations. A linear amplification process was chosen with the intent of increasing the amount of genetic material required for microarray hybridizations. Equal amounts of RNA from the three largest follicles were pooled and a total of five nanogram RNA from the pooled sample was used for RNA amplification. Linear amplification was performed using two 6-hour rounds of T7 RNA polymerase (RiboAmp HS^{Plus} RNA Amplification Kit; Molecular Devices, Sunnyvale, CA, USA) following manufacturer's directions and the produced antisense RNA (aRNA) amount was measured using NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA).

5.3.4 Sample Labeling, Hybridization and Microarray Scanning

For each sample, 2.5 µg of aRNA were labelled using DY-547/647 (Red - CY5 and Green CY3) fluorescent dyes from ULS Labelling Kit (EA-006, Kreatech Diagnostics, Amsterdam, The Netherlands) according to the manufacturer's protocol. With the intent of removing any non-reacted ULS-label material, another round of aRNA purification was performed using the Pico-Pure RNA Isolation Kit but without DNAse I treatment. Pure labeled aRNA was eluded in 11µl elution buffer. Labelling efficiency was measured using the NanoDrop ND-1000. Minimum of 30 pmol/µg of labeling signal was required to proceed with hybridization. A hybridization mix was prepared using 825 ng of each cyanine (Cy3 and Cy5) labeled amplified aRNA, agilent and tomato spikes, nuclease free water, 10X blocking agent and a 25X fragmentation buffer, in a total

volume of 55µl, which was pipetted into the hybridization slides. Three biological replicates in each group (Long FSH vs. Short FSH) were used in the experimental design, in a dye-swap set up. Overall, 6 hybridizations were performed using a custom-build bovine oligo array slide (EmbryoGENE EMBV3 manufactured by Agilent; Design ID: 028298, GEO accession # GPL13226). The slide contained a total of 45,220 oligo nucleotide probes. Each probe had a duplicate and the slide also included Agilent's positive and negative controls in 4x44K format. Oligo sequences were taken from Oligo Microarray Consortium database (BOMC, http://www.bovineoligo.org).

Hybridization were performed (Agilent Technologies Inc., Wilmington, DE, USA) using 2x GEx Hybridization Buffer HI-RPM, at 65^{o}C in preheated oven for 17 hours with rotator speed at 10 rpm. Slides were washed with two buffers from the Gene Expression (GE) wash buffer kit (Agilent technologies Inc. DE USA, catalogue # 5188-5327), according to manufacturer's protocol. Later, slides were dipped in 100% acetonitrile for 10 seconds at room temperature and washed with stabilization and drying solution for 30 seconds at room temperature.

The slides were immediately scanned and visualized using Power scanner (Tecan US Inc, Durham, USA). After image acquisition, scanned images were analyzed and quantified using with Array-Pro Analyzer software (Media Cybernetics, Silver Spring, MD, USA).

5.3.5 Data normalization and Statistical Analysis

Raw signal intensity files were uploaded to EmbryoGENE Laboratory Information Management System (LIMS) and microarray Analysis platform. Quality control of the hybridizations was evaluated using Gydle software (http://www.gydle.com/). Signal intensity data were analyzed using the FlexArray software, version 1.6.1 (Blazejczyk *et al*. 2007). Background subtraction was done with the Flexarray software. If background intensity was higher than the obtained foreground of the median intensity, negative values were replaced with 0.5 as a default. Data were normalized within and between arrays, using Loess and Quantile normalization methods, respectively (Lopez-Romero *et al.* 2010). Linear models for microarray (Limma) was

performed to identify differentially expressed genes in the Long FSH group compared with the Short FSH group (the reference group) (Smyth 2004, Smyth 2005), using a fold-change of 2 and P-value of 0.05 as a threshold. The false discovery rate was estimated by the Benjimeni-Hocheberg method (Li *et al.* 2005), using a fold-change of 2 and a P-value of 0.05 to identify true positive genes.

5.3.6 Functional Annotation and Pathway analysis

The differentially expressed gene list, generated after Limma analysis, was uploaded into Ingenuity Pathways Analysis (IPA Version: 14400082; Ingenuity Systems, www.ingenuity.com) to identify gene networks. Gene networks were used to identify likely biological functions, molecular processes and disorders, and pathways most related to the gene list. IPA analyses are based on human and mouse studies.

5.3.7 Real-time PCR

Seven genes (NTS, PTGS2, PTX3, RGS2, INHBA, CCND2 and LRP8) that were playing a key role in the results and were involved in the hypothesis being generated by the microarray analysis, were selected. Primers were designed using Primer3 v.0.4.0 website (http://frodo.wi.mit.edu/primer3/); analyzed using IDT PrimerQuest tool - Oligo Analyzer website (http://scitools.idtdna.com/analyzer/Applications/OligoAnalyzer/) and blasted using NCBI (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome ; http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthome. Selected primers met the following criteria: 20 to 24 base pairs; 55 to 65 melting temperature; 40 to 60% of CG content; no hairpin, self-dimer or hetero-dimer formation and be specific to the gene of interest. A list of selected primer are presented in Table 1.

After selection, primers were tested by performing RT-PCR of cDNA from pooled granulosa cell samples. After cDNA was amplified during the PCR reaction (see below), the amplicon was run by electrophoresis in a standard 1% agarose gel to determine the size of the band. The band was cut and eluted using a QIAquick Gel

Extraction kit (Cat# 28704, Qiagen, Toronto, ON, Canada), quantified using NanoDrop ND-100 and sequenced (3 x ABI 3730xl Sanger sequencing). Primers were only used if the sequence matched the desired amplicon. The amplicon was also used for generating a standard curve. The standard curve went from 10^{-2} to 10^{-11} ng/µl.

Real time PCR was performed in a Stratagene Mx3005P fast thermal cycler (Applied Bioscience) using SYBR® Green master mix (Applied Bioscience). Cycle threshold (Ct) were recorded; the expression of the interest gene was normalized to geometric mean of UBE2D2, EIF2B2 and SF3A1 using the Relative Expression Software Tool

Table 5.1. Primers used for real-time PCR.

Genes	**Strand**	**Primer sequence**	**Annealing Temperature (°C)**
	Forward	5'-CATGAGATGGCAGTCAATTTGT-3'	53.6
EIF2B2	Reverse	5'-CTTGAACATAGGAGCACAGACG-3'	55.5
	Forward	5'-TGTGTCCCTCTTGCTGAGTTT-3'	56.4
SF3A1	Reverse	5'-ATTCCTGGTTTCACGTCTCCTA-3'	55.5
	Forward	5'-TGGACTCAGAAGTATGCGATGT-3'	55.8
UBE2D2	Reverse	5'-CTTCTCTGCTAGGAGGCAATGT-3'	56.6
	Forward	5'-AGTGTTCCCTCTTGGAAAATGA-3'	60
NTS	Reverse	5'-TCTTCCTGAATCAACTCCCAGT-3'	60.1
	Forward	5'-AAAGCTCTAGGGGGTTCTCG-3'	56.3
PTGS2	Reverse	5'-TGTCAGCACATCCAGGGTAA-3'	56
	Forward	5'-GGCAGACTCACAGGCTTCAATATC-3'	57.6
PTX3	Reverse	5'-CCTTCTCCAGTCTCCCTTTCAACT-3'	58.2
	Forward	5'-AAAGCCGCAGATCACCACAGAA-3'	59.3
RGS2	Reverse	5'-TCCAGCTTGAGACACACCACAT-3'	58.6
	Forward	5'-CGACTTCATCGAACACATCCTTCG-3'	57.7
CCND2	Reverse	5'-CTATTCAGCAGCACCACCTCAATC-3'	57.8
	Forward	5'-ACGCAAAGTTCTCGCAAGCTCA-3'	59.7
LRP8	Reverse	5'-TGCCATTTCCTCCTCAAACAGG-3'	57.6

	Forward	5’-CCAAAGGATGTACCCAACTCTC -3’	59.8
INHBA	Reverse	5’-GTCCGATGTCGTCCTCTATCTC -3’	60.7

5.3.8 Radioimmunoassays

Follicular fluid levels of estradiol and progesterone were measured using radioimmunoassay. Long FSH group was compared with Short FSH group. Slaughterhouse ovaries were used to obtain a charcoal-extracted pool of follicular fluid, which was used to prepare the standards and dilute follicular fluid samples. Standard curve ranged from 5 to 1000 pg/ml for estradiol and 0.1 to 40 ng/ml for progesterone. Samples were diluted using the charcoal-extracted pooled follicular fluid so that hormone concentrations fell within the limits of the standard curve and samples were assayed in duplicates. Estradiol was measured with a modified human double-antibody RIA Kit (Catalog # KE2D1, Coat-A-Count; Siemens Healthcare Diagnostics Inc.; Mississauga, ON, Canada), dilutions ranged from 1:25 to 1:500. Progesterone was measured using commercial radioimmunoassay kit (Catalog # TKOP1, Coat-A-Count; Siemens Healthcare Diagnostics Inc.; Mississauga, ON, Canada) and all samples were diluted 1:10. All samples for progesterone analysis were analyzed in a single assay only and the intra-assay coefficient of variation was 5.6%. Estradiol was measured in two different assays and the intra-assay coefficient of variation was 11%, while inter-assay coefficient of variation was 8.1%. Hormone data were analyzed by analysis of variance using a general linear model procedure (GLM) (SAS Learning Edition 4.1; SAS Institute; Cary, NC, USA) to compare follicular fluid from Long FSH vs. Short FSH group (7 days vs 4 days superstimulation; respectively).

5.4 Results

5.4.1 Differential gene expression profile

Short FSH group was used as the reference group for all comparisons. There are a total of 1031 differently expressed genes in Long FSH group compared with Short FSH (Figure 5.2). Out of these, 416 genes have a significantly lower expression in Long FSH

group (i.e. down-regulated genes), while 615 genes have a significantly higher expression (i.e. upregulated genes) when compared with Short FSH (fold change =2 and P value = 0.05). One hundred and forty-two of these transcripts are novel. Table 5.2 lists the 10 most up- or down-regulated granulosa cell gene transcripts after Long FSH (i.e. 7 days superstimulation) compared to Short FSH group (i.e. 4 days superstimulation).

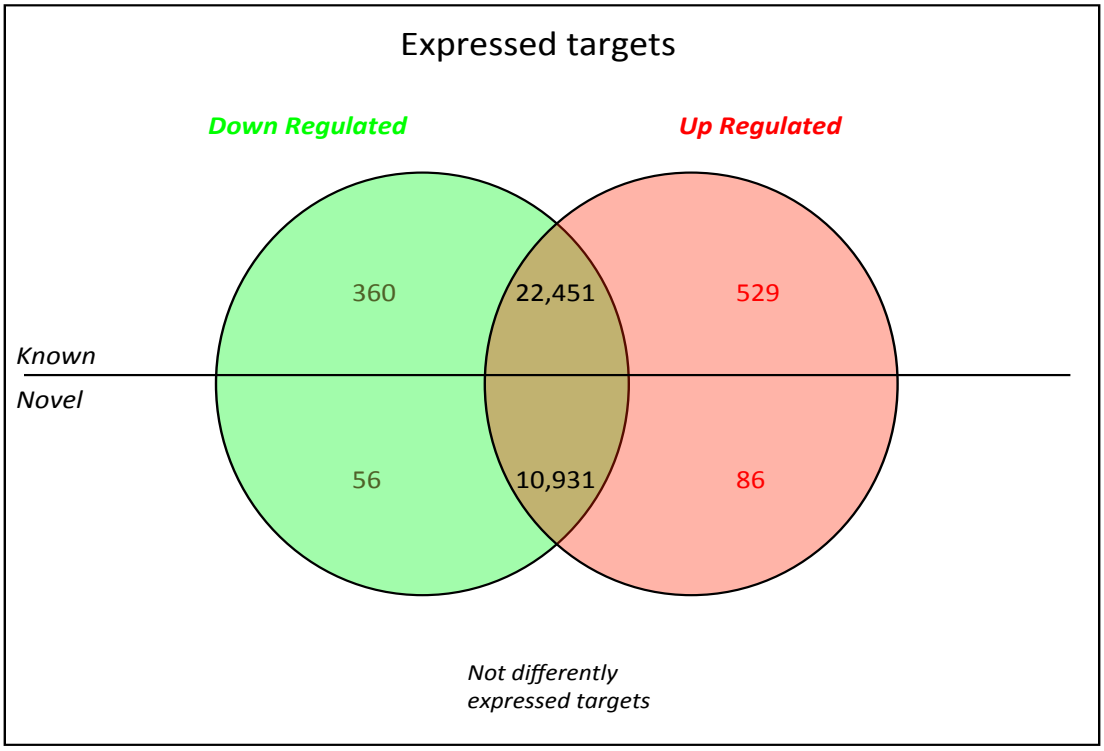


Figure 5.2. Venn diagram summarizing microarray analysis of granulosa cells after a Long (7-day) compared to Short (4-day; reference group) FSH treatment. Cells were obtained from the FSH-stimulated follicles 24 hr after exogenous LH treatment in both groups. Limma was used for statistical analysis and up- and down-regulated targets was determined using fold change of 2 and P value of 0.05.

Table 5.2. Top 10 up and down-regulated bovine genes in granulosa cells after a Long vs. Short duration of growing phase of dominant follicle under superstimulation.

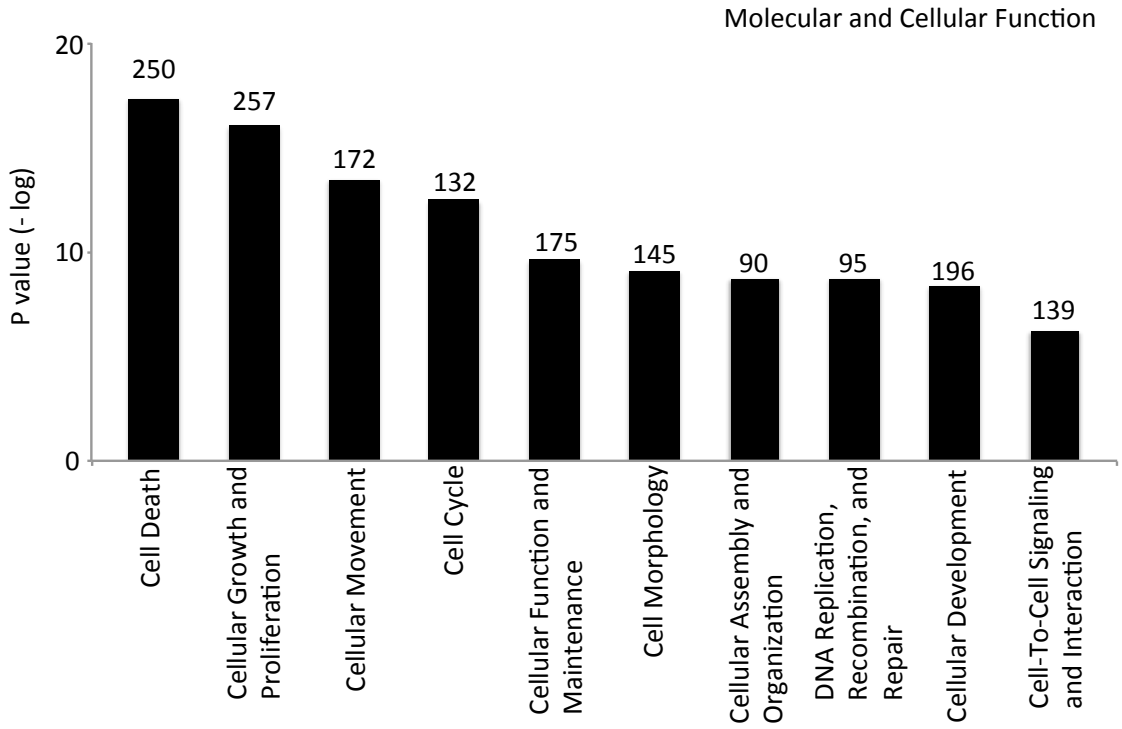
Genes	Description	P Value	Fold

			change
UpRegulated			
PTX3	Pentraxin 3	$3.7x10^{-13}$	5.904
VNN3	Vanin 3	$3.3x10^{-11}$	5.281
POSTN	Periostin, Osteoblast specific factor	$1.4x10^{-07}$	4.241
PTGS2	Prostaglandin endoperoxide synthase 2	$1.5x10^{-07}$	4.182
GRIA3	Glutamate receptor, ionotropic, AMPA3	$8.4x10^{-07}$	4.175
RGS2	Regulator of G protein signaling 2	$2.9x10^{-06}$	3.702
GFRA1	GDNF family receptor alpha 1	$7.5x10^{-05}$	3.679
PLA2G4A	Phospholipase A2 group Iva (cytosolic, calcium dependent)	$4.4x10^{-07}$	3.605
CRISPLD2	Cysteine rich secretory protein LCCL domain containing 2	$2.9x10^{-05}$	3.604
GIMAP4	GTPase IMAP family member 4	$9.0x10^{-12}$	3.545
Down-Regulated			
INHBA	Inhibin beta A	$2.0x10^{-07}$	-3.978
LRP8	Low density lipoprotein receptor-related protein 8	$1.4x10^{-07}$	-3.784
CCND2	Cyclin D2	$5.9x10^{-09}$	-3.437
SRGN	Serglycin	$1.2x10^{-05}$	-3.364
BEX2	Brain expressed X-linked 2	$3.3x10^{-07}$	-3.055
INHBB	Inhibin beta B	$2.9x10^{-07}$	-3.031
TNNI3	Troponin 1 type 3	$6.9x10^{-08}$	-2.82
CYP51A1	Cytochrome P450, family 51, subfamily A, polypeptide 1	$1.5x10^{-09}$	-2.593
CALB2	Calbindin 2	$1.6x10^{-07}$	-2.522
ENPEP	Glutamyl aminopeptidase (aminopeptidase A)	$2.2x10^{-08}$	-2.473

5.4.2 Function, network and pathway analyses

The gene list obtained after limma analysis was up-loaded in Ingenuity® , and function, network and pathway analyses were performed. The top 10 functions and the top 10 pathways are shown in figure 5.3 and 5.5. A network with highly differently expressed genes was generated and is shown in figure 5.4. In this network, most granulosa cell genes are markers of follicle LH responsiveness, oocyte nuclear maturation and oocyte competence

A)



B)

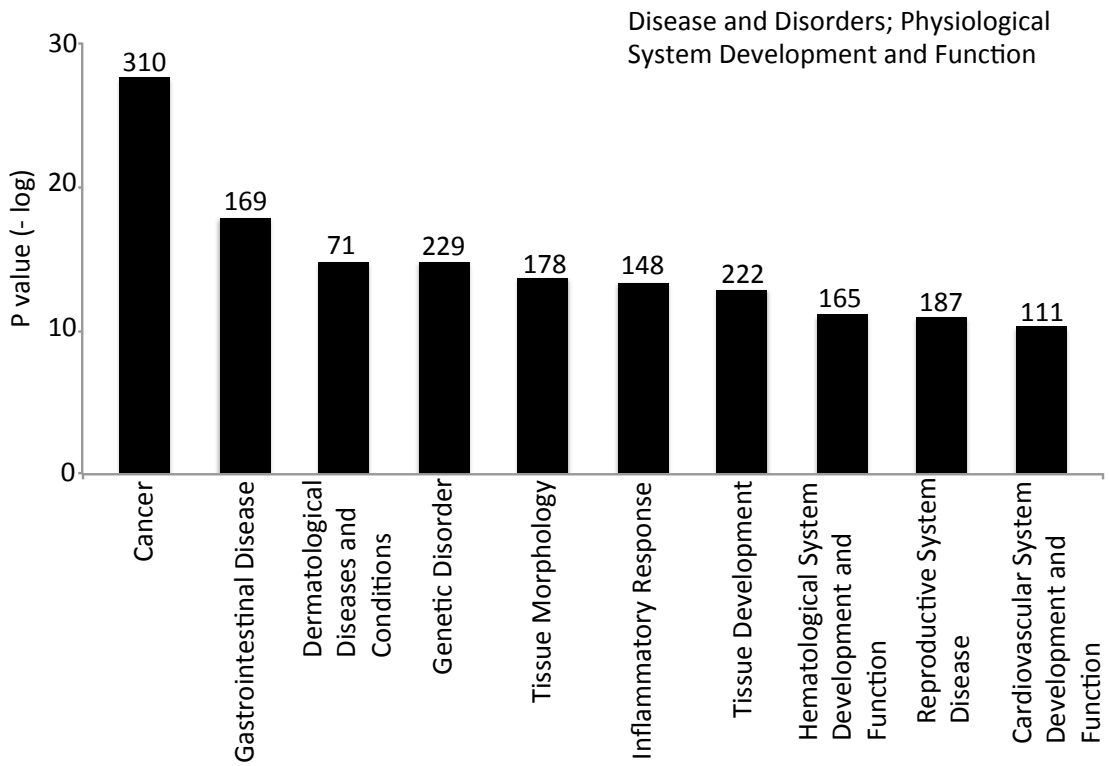


Figure 5.3. Function analysis of gene expression of granulosa cells after a Long (7-day) compared to Short (4-day; reference group) FSH treatment based on log P-value. The higher the log P-value (taller bars) the more significant the function is. Numbers on the top of the bars indicate number of genes involved with each function. Top 10 molecular and cellular functions (A) and top 10 disease and disorders/ physiological function (B) are illustrated.

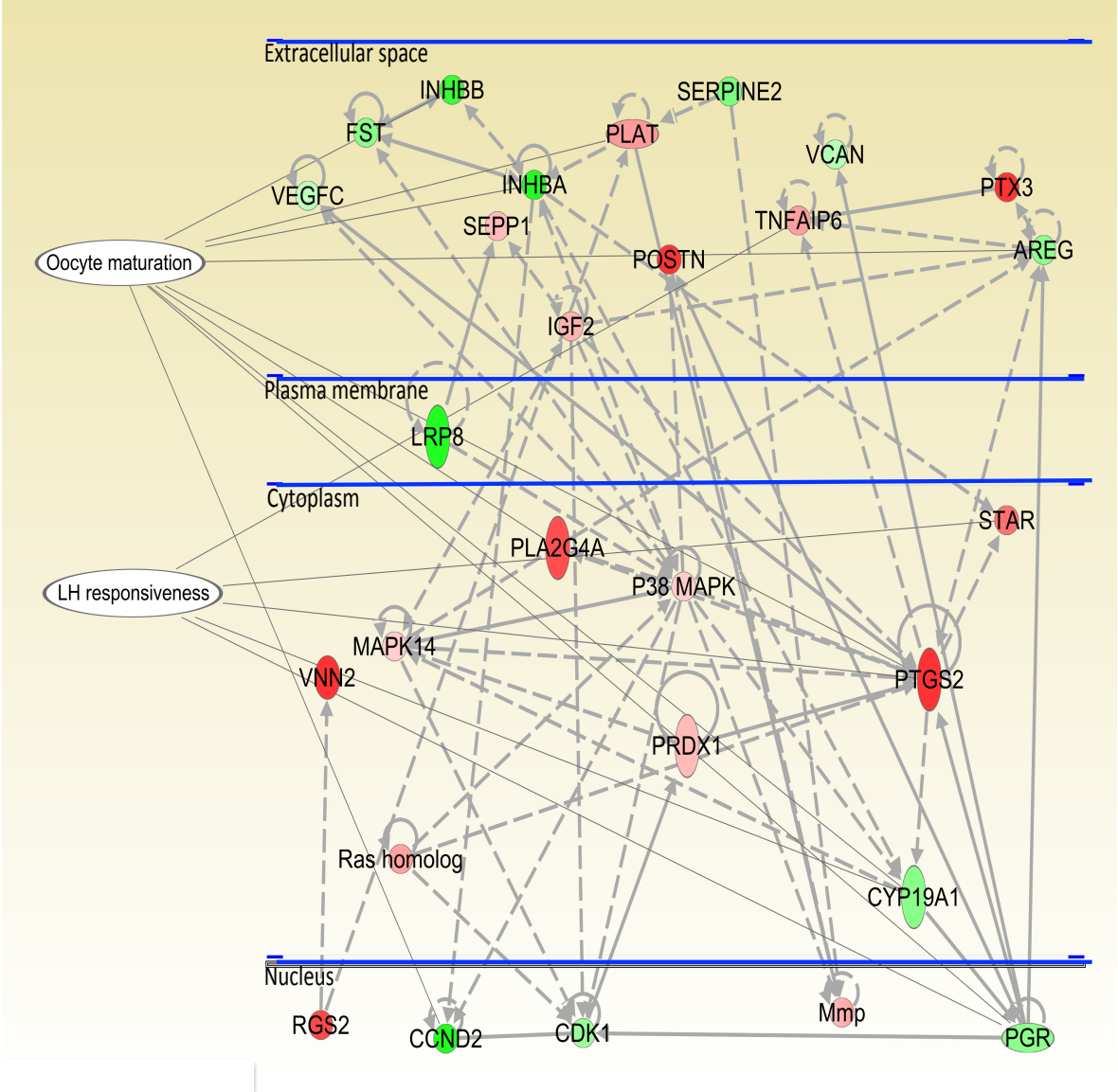
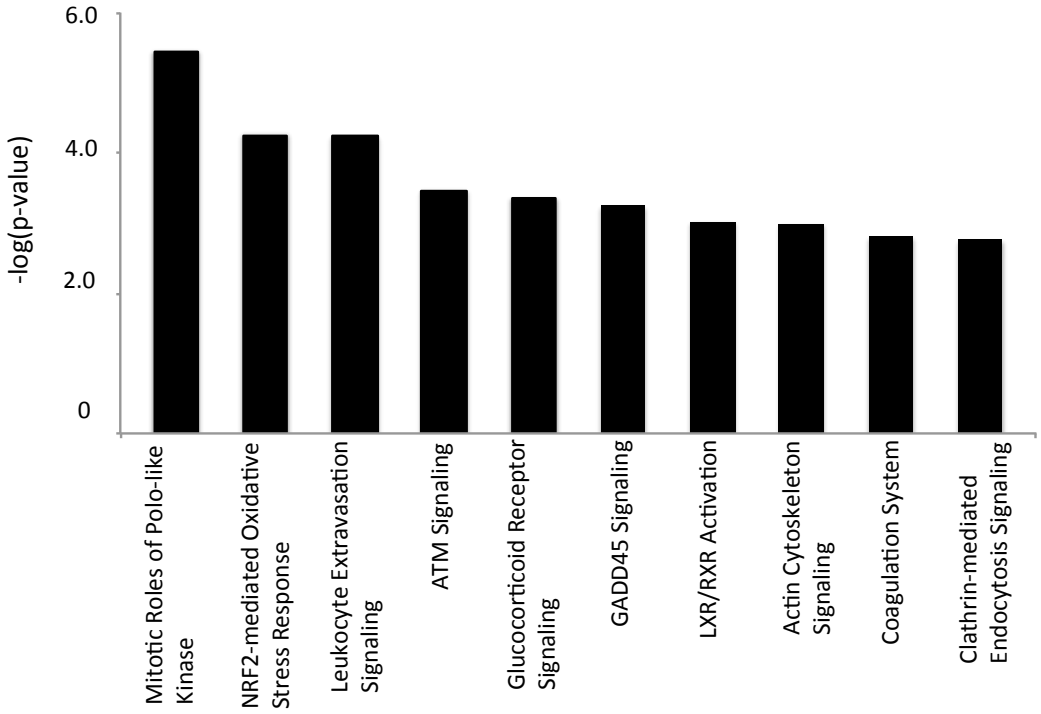


Figure 5.4. Network of genes up- or down-regulated in granulosa cells after Long vs. Short duration of growing phase of dominant follicles (4-day FSH vs. 7-day FSH treatment). Cells were obtained from the FSH-stimulated follicles 24 hr after exogenous LH treatment in both groups. All genes involved in this network are markers of follicle LH responsiveness and oocyte nuclear maturation. The differences in color intensity of molecules show the degree of up- or down-regulation. Green molecules indicate down-regulated genes, while red molecules indicate upregulated genes. The genes found to be upregulated are: Regulator of G-protein signalling 2 (RGS2); Matrix metalloproteinase (Mmp); Vanin (VNN2); GTPase Rho (Ras (homolog)); Peroxiredoxin 1 (PRDX1); Prostaglandin endoperoxide synthase 2 (PTGS2); Steroidogenic acute regulatory protein

(STAR); Mitogen activated protein kinase P38 (P38 MAPK); Mitogen activated protein kinase 14 (MAPK14); Phospholipase A2, group IVA (PLA2G4A); Insulin-like growth factor 2 (IGF2); Selenoprotein P plasm 1 (SEPP1); Periostin osteoblast specific factor (POSTN); Tumor necrose factor, alpha-induced protein 6 (TNFAIP6); Pentraxin 3 (PTX3) and Plasminogen activator (PLAT). The genes found to be down-regulated are: Inhibin beta B (INHBB); Follistatin (FST); Serpin peptidase inhibitor clade E member 2 (SERPINE2), Vascular endothelial growth factor C (VEGFC), Inhibin beta A (INHBA); Versican (VCAN); Amphiregulin (AREG); Low-desity lipoprotein receptor related protein 8 (LRP8); Cytochrome P450 family 19 subfamily A polypeptide 1 (CYP19A1); Cyclin D2 (CCND2); Cyclin dependent kinase 1 (CDK1); Progesterone receptor (PGR).

A)



B)

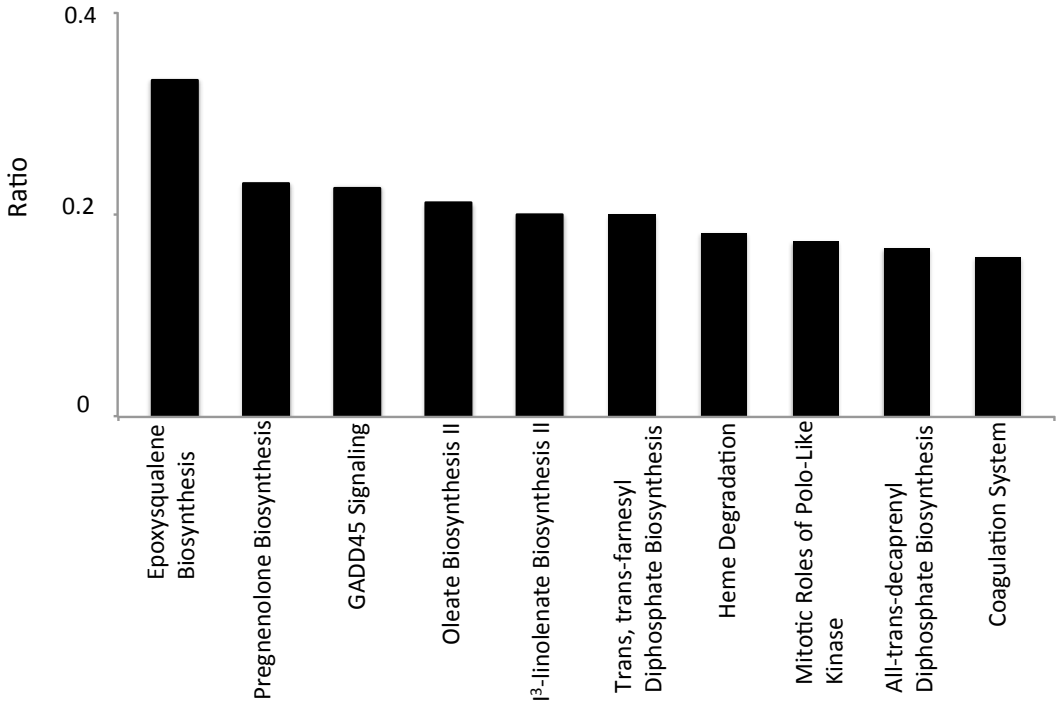


Figure 5.5. Canonical pathways analysis of gene expression of granulosa cells after a Long (7-day) compared to Short (4-day; reference group) FSH treatment, using Ingenuity Pathway Analysis software (IPA). A) Top 10 pathways analysis based on -$\log_{10}$ p-value. B) Top 10 pathway analysis based on a score ratio. The score ratio was calculated by IPA and is the number of differentially expressed molecules in the gene list/ number of genes known to be involved in the pathway.

5.4.3 Real- Time PCR validation

Based on microarray data and functional analysis, a total of 7 genes (NTS, PTGS2, PTX3, RGS2, INHBA, CCND2 and LRP8) were selected for validation with RT-PCR. Those selected genes were quantified in three independent biological replicates from Long and Short FSH groups. Results showed (Figure 5.6) positive validation of differential expressed for 4 out of 7 genes (NTS, PTGS2, PTX3 and RGS2) using 90%

confidence level (P value ≤ 0.1). Although three genes did not show a statistical significance (INHBA, CCND2, and LRP8), they all follow the same trend as microarrays dataset (Figure 5.6).

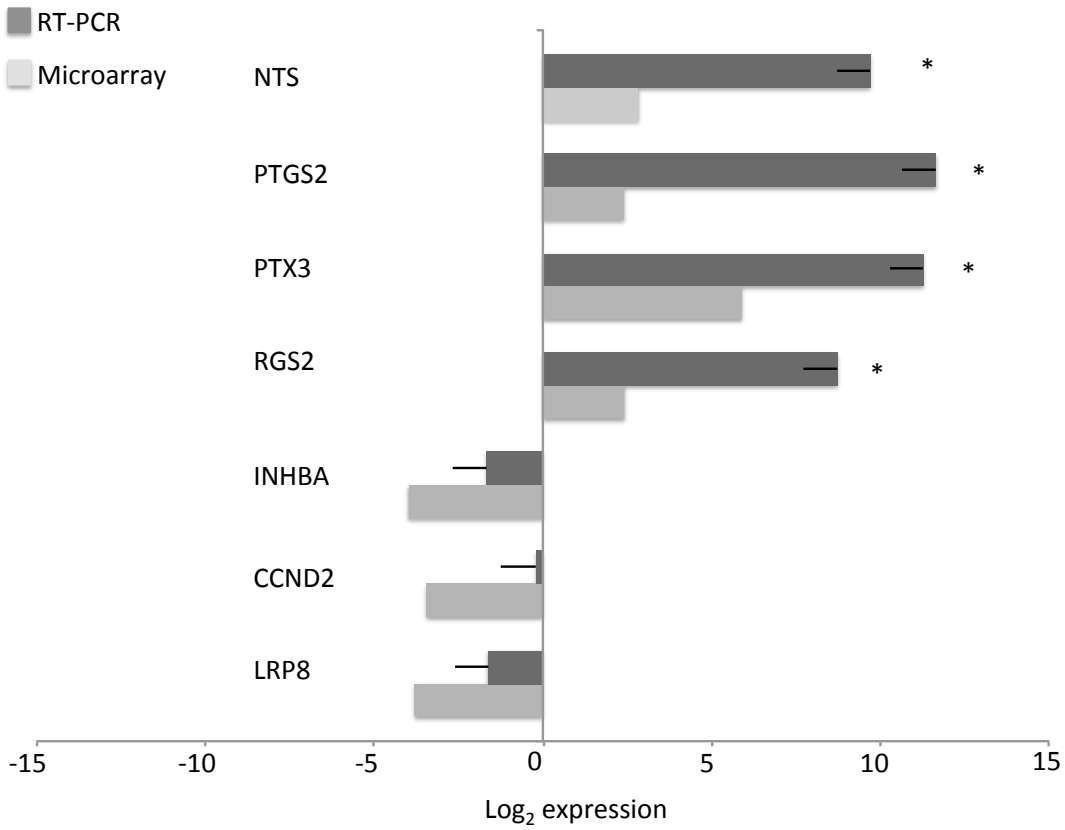


Figure 5.6 Quantification (log_2 of fold-change; mean ±SEM) of the mRNA profiles in granulosa cells after a Long (7-day) compared to Short (4-day; reference group) FSH treatment. Cells were obtained from the FSH-stimulated follicles 24 hr after exogenous LH treatment in both groups RT PCR data analysis was performed using REST 2009 program. Light grey bars represent expression of transcript in microarray experiment while dark grey bars represent expression of the same transcript obtained by RT-PCR. Asterisk (*) above each bar represent statistical difference between Long FSH in relation to the Short FSH in RT-PCR experiments.

5.4.4 Follicular Fluid Analysis

Follicular fluid from follicles of the Long FSH group had lower levels of estradiol compared with follicles of the Short FSH group (27.1 ± 7.6 and 153.8 ± 32.7 ng/ml; mean±SEM, respectively; P=0.001). Follicular fluid progesterone levels were higher in the Long FSH group compared with the Short FSH group (212.9 ± 24.5 and 99.9 ± 19.7 ng/ml; respectively; P=0.001). Estradiol:progesterone ratio was lower in follicles of the Long FSH group compared with the Short FSH (0.13 ± 0.04 and 3.5 ± 0.8, respectively; P=0.0006).

5.5 Discussion

Microarray-based comparisons of mRNA transcripts of granulosa cells after standard 4-day FSH treatment versus 7-day treatment point out to two important postulates: 1) Extending the superstimulation protocol by 3 days activated molecular mechanisms of LH-responsiveness; 2) follicles from Long FSH group had increased expression of markers of oocyte competence in the granulosa cells. Therefore, the Long FSH are in the right stage of development based on ability to respond to exogenous LH surge; opposed to follicles from Short FSH (the reference group). The 7-day superstimulatory treatment is an attractive alternative to obtain healthier follicles and oocytes.

LH surge is an important event that triggers ovulation and culminates in meiotic resumption by the oocyte. At the molecular level, the peak in LH is accompanied by many changes in gene expression and the specific genes known to be influenced by LH surge are usually referred as “markers of LH responsiveness”. In our study, VNN, POSTN, PLA2G4A, GTPase, Cysteine (Figure 5.4) were upregulated in Long FSH group – these genes have been described as markers of LH surge (Gilbert *et al.* 2011, Gilbert *et al.* 2012) Conversely, most of the down-regulated genes (CYP19A1, LRP8, CJA1, INHBA and SERPINE2; Figure 5.4) are markers of pre LH surge or are known to be down-regulated after LH surge (Ndiaye *et al.* 2005). It is interesting to note that animals

were ovariectomized 24h post-LH treatment; therefore follicles from Long FSH group are responding better to exogenous LH surge than those from the standard 4-day protocol.

Pentraxin 3 (PTX3) is the most upregulated gene in this study and its expression increases after the LH surge (Agca *et al.* 2006). PTX3 is also considered a marker of high fertility (Zhang *et al.* 2005, Wu *et al.* 2012), suggesting that Long FSH treatment would probably result in healthier oocyte. A previous study (Chapter 2) compared the effect of similar superstimulation protocols (Long vs Short FSH) to evaluate oocyte competence after in vitro fertilization. The quality of oocytes collected was not significantly different, however Long FSH resulted in 2.5 more transferable embryos and a greater proportion of zygotes developing into embryos (Chapter 2). Similarly, when Endoplasmic Reticulum (ER) Stress was artificially activated in Cumulus-Oocyte Complexes, the secretion of Pentraxin-3 was significantly reduced; in vitro fertilization rates was reduced, and embryos were slower to develop to blastocysts(Wu *et al.* 2012). Perhaps, a greater expression of pentraxin 3 is linked to a greater embryonic development obtained after a Long FSH protocol in that earlier study (Chapter 2).

The LH surge also influences the production of prostaglandins by follicular cells that are crucial during the ovulatory process. Prostaglandins biosynthesis depends in the initial release of arachidonic acid from membrane phospholipids and their production is increased by LH surge (Diouf *et al.* 2006). PLA2G4A gene expression is responsible for this LH-stimulated mobilization of arachidonic acid (Duffy *et al.* 2005), via the adenylyl cyclase/cAMP pathway (Diouf *et al.* 2006), while PTGS2 (or COX 2) converts arachidonic acid to prostaglandin (Shimada *et al.* 2006). Many studies show the lack or disturbance of ovulation when PTGS2 expression is inhibited (Davis *et al.* 1999, Pall *et al.* 2001, Bridges *et al.* 2006, Hester *et al.* 2010). PTGS2 is highly expressed in bovine preovulatory follicles after endogenous LH surge and its follicular fluid concentration is high 0 to 24h after estrus detection (Liu *et al.* 1997). PTGS2 also directs oocyte maturation by differentially influencing multiple signaling pathways as cAMP-dependent protein kinase, MAPK, NF-kappaB and phosphatidylinositol 3-kinase/AKT pathway (Takahashi *et al.* 2006). The expression of genes for both enzymes (PLA2G4A and

PTGS2) was upregulated in Long FSH group which indicate that follicles of this group were more mature, close to ovulation and better responsive to LH than those after standard FSH treatment.

Genes encoding the low-density lipoprotein receptor-related protein 8 (LRP8), cyclin D2 (CCND2) and cytochrome P450 family51 subfamilyA polypeptide1 (CYP51A1) are all known to be down-regulated after LH surge (Agca *et al.* 2006, Fayad *et al.* 2007); (Vaknin *et al.* 2001). Interestingly, all of these were down-regulated in Long FSH group compared to 4-day FSH treatment group. CCND2 functions as a regulatory subunit of cyclin dependent kinase 4 or 6 (CDK 4 or 6) whose activity is required for cell cycle G1/S transition. Cyclin D2 mRNA is localized to granulosa cells of growing follicles and CCND2 mRNA is induced by FSH but rapidly inhibited by LH (Robker & Richards 1998b, Robker & Richards 1998a). (Robker & Richards 1998b). When ovulatory doses of human LH were administered to rats, cyclin D2 mRNA and protein were rapidly decreased and were undetectable within 4 h. Presence of CCND2 in human cumulus cells at the time of oocyte retrieval is also suggested to be a marker for lower embryo development and consequently low fertility (van Montfoort *et al.* 2008). The fact that is CCDN2 gene is expressed at higher level after standard FSH treatment (i.e. down-regulated in Long FSH group) demonstrate that extending the super stimulation protocol by 3 days could be beneficial to follicular and oocyte health.

Follicular cells and oocytes from superstimulated follicles are commonly used in applied research programs (review in (Mapletoft & Bo 2011) and (Mapletoft *et al.* 2002)). However, until recently there was little information in how and to what extend does superstimulation affect bovine follicular genomics and if follicles from a superstimulation pool were really comparable to dominant follicle from a natural cycle. A previous study from our lab (Chapter 3) tested the effect of superstimulation in gene expression of granulosa cells from 4-day standard superstimulation protocol in comparison with dominant follicles from regular cycle. It was concluded that follicles that undergo a 4-day superstimulation were lagging behind in maturation and differentiation since most of the upregulated genes were markers of the follicular growing

stage. The present study used a similar 4 days standard superstimulation protocol as a reference to compare to an extended protocol (7 days; i.e., 3 days longer than the reference used). It is interesting to note that the same markers for growing follicles are now down-regulated in the extended protocol suggesting that the extra 3 days allow follicles to reach the optimal stage of development. This protocol is based on the life span of a 2-wave animal where a single ovulatory (dominant) follicle grows for 3 days longer when compared to the one from a 2-wave cycle (Ginther *et al.* 1989c). Although it has been suggested that animals that have 3-waves would have a higher fertility (Ahmad *et al.* 1997, Townson *et al.* 2002), results of a large study showed no difference between 2- and 3- wave animals (Dias *et al.* 2012b). Analogous to ovulatory follicles from 2-wave animals, extending the superstimulation protocol by 3 days allowed the follicles to reach the optimal maturity.

We were also interested to examine if follicles from Long FSH would express genes similarly to a persistent follicle or if genes related to atresia and apoptosis were upregulated in the Long FSH group. We found only few apoptotic genes being expressed at higher levels after 7-day FSH treatment, e.g., Caspase 8 was upregulated in this group. Sequential activation of caspases plays a central role in the execution-phase of cell apoptosis. Although follicular fluid from Long FSH had significant lower E2:P4 ratio than after standard FSH treatment, this ratio was much greater than subordinate follicles (Singh *et al.* 1998), indicating that follicles from Long FSH were still estrogen active (E2:P4 ratio >1) and not fully atretic. Early atresia is not considered detrimental to oocyte competence rather; early atresia is the best option for in vitro conditions (Blondin & Sirard 1995, Blondin *et al.* 1997a, Salamone *et al.* 1999). (Blondin *et al.* 1997a, Barnes & Sirard 2000). Perhaps, early atretic changes are beginning to occur in the Long FSH group whereas follicles in the standard FSH treatment group are still in the growing stage and less differentiated. Our results also indicate that granulosa cells/follicles from the Long FSH group were behaving somewhat like persistent follicles. Aminoacid transporter A2, Aurora Kinase family (A and B), Frustose – 1,6 bisphosphatase and Malate dehydrogenase were all down- regulated in persistent follicles and in Long FSH (present study). Likewise, Glutathione S-transferase Isoform was up- regulated in both

persistent follicles and the Long FSH group. Perhaps the extra 3 days may be the limit of a healthy follicle.

In conclusion, based on transcriptome information, extending FSH treatment by 3 days allowed the granulosa cells to differentiate better and respond optimally to LH stimulus. The extended protocol also activates markers for ovulation and oocyte competence.

Acknowledgements

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CHAPTER 6

GRANULOSA CELL GENE EXPRESSION AFTER FSH WITHDRAWAL DURING SUPERSTIMULATION TREATMENT IN CATTLE

Dias F.C.F., Khan M.I., Sirard M.A., Adams G. P., Singh J.

Relationship of this study to the dissertation

Follicular aging refers to the phenomenon when a dominant follicle in the ovulatory wave is allowed to grow for longer period before it ovulates. We used a superstimulation model to test the effect of follicular aging in oocyte competence after *in vitro* fertilization (Chapter 3) and in gene expression of granulosa cells (Chapter 5 and the present chapter). The microarray comparison addressed in Chapter 5 is a 4-day short superstimulation versus a 7-day long superstimulation (the protocol for follicular aging). The comparison addressed in this Chapter is the use of a long 7-days superstimulation protocol with continuous FSH support or after FSH withdrawal.

6.1 Abstract

A short period of FSH starvation (withdrawal of FSH) during superstimulation treatment may be beneficial to oocyte competence, while 96 or 144 hours of FSH starvation leads to loss of ovulation capability. The objective was to evaluate the effect of FSH starvation in gene expression of granulosa cells. Cows were allocated randomly to an FSH starvation group and a control group (reference group; 7 days superstimulation) (n=6/group). A new follicular wave was induced 5-8 days after ovulation by ablation of ≥5 mm follicles using transvaginal ultrasound-guided follicle aspiration, and a progesterone-releasing device (CIDR) was placed in vagina. The FSH starvation group was given eight injections of 25mg FSH at 12-h intervals starting from the day of wave emergence (Day 0), whereas the control group was given fourteen injections. Both groups were given prostaglandin $F_{2\alpha}$ im twice, 12 h apart, in Day 6 and CIDR devices were removed at the second injection; 25 mg pLH was given im 24 h after CIDR removal, and cows were ovariectomized 24 hour later. Granulosa cells were collected for RNA extraction, amplification and microarray hybridization or RT-PCR (n=3 cows each for microarray analysis and RT-PCR validation). To translate microarray results to a physiological context, a list of differentially expressed transcripts were biologically annotated. A total of 396 genes were down-regulated and 275 genes were upregulated in the FSH starvation group compared to controls. To validate the microarray results, 7 genes were selected for real time-PCR (NTS, PTGS2, PTX3, RGS2, INHBA, VNN1 and LRP8). Five of the 7 genes (71.4%) were either statistically different ($P<0.05$) or had a tendency to differ ($P<0.1$); Gene annotation analysis showed the FSH starvation group had decreased expression of genes related to cell growth, markers of LH responsiveness and ovulation, and markers of oocyte quality and embryo development. Results of microarray analysis after FSH starvation provided rationale for the following hypotheses: 1) FSH starvation results in growth arrest; 2) FSH starvation alters genes involved in LH responsiveness; 3) FSH starvation disturbs expression of genes in the prostaglandin-signaling pathway causing ovulation failure; and 4) FSH starvation affects the expression of granulosa cell gene markers of oocyte quality and embryo development.

Keywords: cattle, FSH, follicular growth, granulosa cells, mRNA expression, trancriptome analysis, superstimulation.

6.2 Introduction

Follicle stimulation hormone (FSH) promotes follicular growth (Adams *et al.* 1992a, Adams *et al.* 1992b) by activating its receptor in the plasma membrane of granulosa cells (van Koppen *et al.* 2013). High levels of FSH induce granulosa cell proliferation during early folliculogenesis, the expression of LH receptors in granulosa cells from both preantral and preovulatory follicles (Adriaens *et al.* 2004, Sasson *et al.* 2004) and prevent follicular atresia (Franchimont *et al.* 1988). FSH also stimulates the mRNA expression of P450 cholesterol side chain cleavage enzyme (Urban *et al.* 1991, Silva *et al.* 2006). This enzyme is part of the steroidogenic pathway and is responsible for the synthesis of pregnenolone from cholesterol. Production of estradiol and inhibin, and the acquisition of LH receptors in the granulosa cells of the future dominant follicle are all key parts of the follicle selection process (Adams *et al.* 1993b) and the post-surge decline in FSH is temporally associated with selection (Adams *et al.* 1992b, Fortune *et al.* 2004). FSH also affects oocyte function and developmental competence through granulosa cell signalling (Ali & Sirard 2005, Hunzicker-Dunn & Maizels 2006). Studies in mouse have shown that FSH-beta and FSH-receptor knock-out females are infertile or have disturbed estrous cycles with anovulatory follicles (Kumar *et al.* 1997, Danilovich *et al.* 2000). Thus, FSH surges during the estrous cycle are crucial for follicular health and oocyte competence.

Continuous exogenous stimulus of FSH starting at the beginning of a follicular wave results in growth of multiple ovulatory follicles in monovular species (ovarian superstimulation). Ovarian superstimulation is well established as a tool for assisted reproductive technology in cattle. However, there are still many unanswered questions regarding ovarian superstimulation. The variation in ovarian response to superstimulatory treatment among cows is high and many protocols have been developed in an attempt to

optimize the superstimulatory response (Adams 1994, Mapletoft *et al.* 2002, Mapletoft & Bo 2011). Recent studies have shown an increased superstimulatory response by extending the standard 4-day FSH protocol to 7 days (Dias *et al.* 2012a, Garcia Guerra *et al.* 2012). One study (Garcia Guerra *et al.* 2012) showed increased ovulation within 36 hours post LH with the extended protocol; however, no difference in oocyte competence was detected. At the molecular level, preovulatory follicles from the standard 4-day protocol appeared to exhibit a delayed response to LH when compared to the dominant follicle of a natural unstimulated cycle (Chapter 4). This delay in response to LH was corrected when FSH treatment was extended to 7 days (Chapter 5). However, it is not clear if the improvement of follicular cells to respond to LH is attributable to the extra time or extra FSH in the extended protocol.

Some studies showed that a period of FSH starvation (coasting) at the end of the superstimulation protocol is beneficial to oocyte competence using pre-LH collection and in vitro maturation (Blondin *et al.* 1997b, Sirard *et al.* 1999, Blondin *et al.* 2002). An increased in the proportion of oocytes that developed into embryos was reported using 48 hours of coasting (Blondin *et al.* 1997b). However a higher blastocyst rate (80%) was obtained when the 48 hours of coasting was preceded by an extended superstimulation protocol (6 vs. 4 doses of FSH) (Blondin *et al.* 2002). In a more recent study (Nivet *et al.* 2012), 92 hours of FSH starvation resulted in an increase in the number of larger follicles (>9mm); however, the blastocyst rate was reduced when compared to 44 and 68 hrs of FSH starvation. In those studies , higher levels of progesterone were maintained due to the presence of a corpus luteum; so basal LH maintained follicular growth until 92 hrs.

Previous studies from our laboratory showed that FSH starvation resulted in an ovulation failure rate of 100% if the follicles grew under a sub-luteal progesterone environment (Jaiswal R.S. *et al.* 2006) and an ovulation failure rate of about 50% when progesterone was kept at diestrous levels (Dias *et al.* 2012a). Due to the lack of ovulation, oocyte competence could not be assessed after insemination in vivo in those studies. When oocytes were obtained by ultrasound-guided follicle aspiration 24 hours after LH treatment given 96 hrs after FSH withdrawal, it resulted in 96% fertilization failure in vitro (which preclude the capacity to assess competence to develop; Chapter 3). Based on those studies there are needs to further understand the effect of FSH starvation as many

questions still remain unanswered: How does FSH starvation affect ovulation? What change does FSH starvation induce in oocytes to compromise fertilization rate? Are FSH-deprived follicles responsive to LH? Do these follicles behave as persistent follicles? A detailed comparison of gene expression of granulosa cells between 7-day superstimulation with 96 hrs of FSH starvation and a 7-day superstimulation with continuous FSH support may help to answer some of these questions.

The objective of the study was to evaluate the effect of FSH starvation in gene expression in granulosa cells. We tested the hypothesis that FSH starvation will induce changes in gene expression profiles related to growth arrest, and apoptosis when compared to continuous FSH support. Moreover, we expect markers of LH responsiveness to be turned off by FSH starvation.

6.3 Material and Methods

6.3.1 Animals and Treatments

Cross-bred beef cows (n=12), weighing 515 to 795 kg, and maintained in outdoor corrals, were used. All procedures were conducted in accordance with the guidelines of the Canadian Council in Animal Care and were approved by University of Saskatchewan Protocol Review Committee.

Ovulation was synchronized by 2 im treatments with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; 500 µg of cloprostenol; Estrumate, Schering-Plough Animal Health, Pointe-Claire, PQ, Canada) at 14-day intervals. Daily ultrasonography was performed from the time of the second $PGF_{2\alpha}$ injection until ovulation was detected. Five to 8 days after ovulation, ablation of all follicles ≥5 mm in diameter was performed using transvaginal ultrasound-guided follicle aspiration with the intent to synchronize the emergence of a new follicular wave 1 day later (Bergfelt DR *et al.* 1994). An intravaginal progesterone-releasing device, CIDR (Pfizer Canada Inc., QC, Canada), was placed in the vagina immediately after follicle ablation.

The cows were allocated randomly to two groups (n=6 per group; Figure 6.1): a) FSH starvation group; and b) reference group (7 days superstimulation). Starting 1 day after follicle ablation, i.e., in the day of wave emergence (Day 0), cows in the FSH

starvation group were given 8 im injections of FSH (Folltropin-V; Bioniche Animal Health, Belleville ON, Canada; each equivalent to 25 mg of NIH-FSH-P1) at 12-hour intervals, whereas cows in the reference group were given 14 im doses of FSH (each dose equivalent to 25 mg of NIH-FSH-P1). Cows in both groups were given two luteolytic doses of $PGF_{2\alpha}$ 12 h apart in Day 6, and the CIDR was removed at the time of the second $PGF_{2\alpha}$ treatment. Cows were given 25 mg pLH im (Lutropin-V, Bioniche Animal Health) 24 hours after CIDR removal and were ovariectomized 24 hours after pLH treatment..

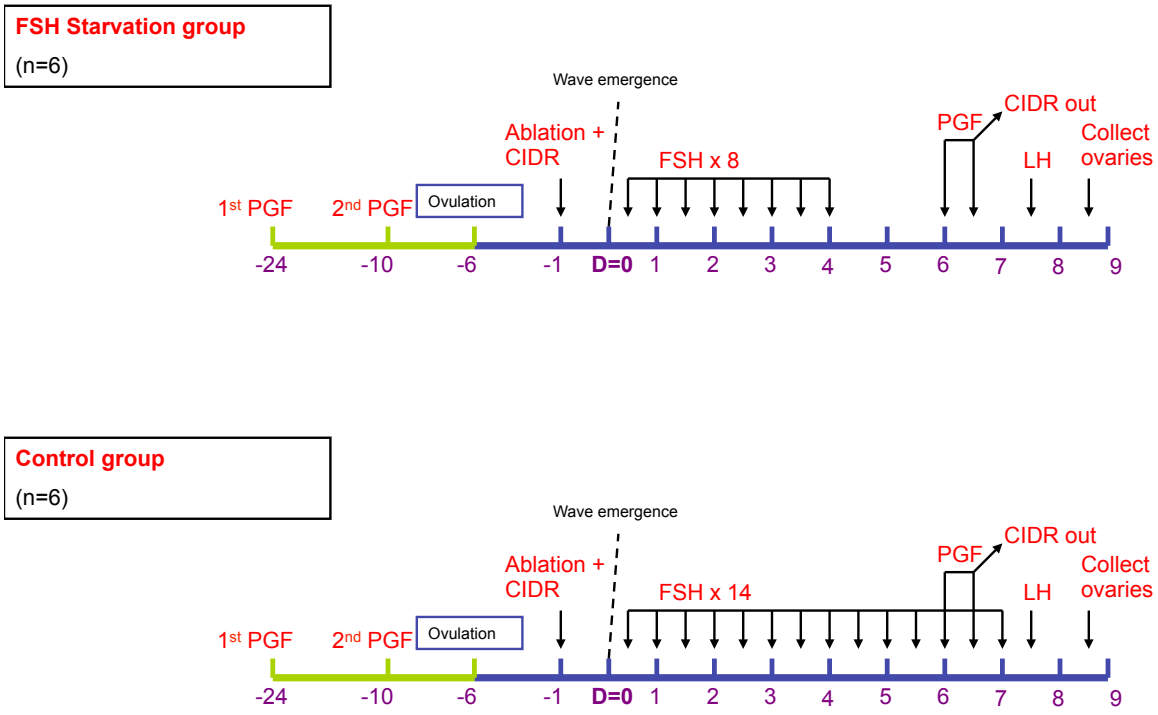


Figure 6.1. Experimental protocol used to test the gene expression of granulosa cells after withdrawl of FSH during superstimulation treatment in cattle. Five to 8 days after ovulation, follicle ≥5 mm were ablated and a CIDR was placed in the vagina. FSH treatment started at wave emergence (Day 0). The FSH starvation group was given 8 im injections of FSH at 12-hour intervals, whereas cows in the reference group were given 14 doses of FSH. $PGF_{2\alpha}$ treatments were given in Day 6 and 6.5, and the CIDR was

removed; LH was given 24 hours after CIDR removal. Cows were ovariectomized 24 hours after exogenous LH treatment.

6.3.2 Tissue Collection

Ovariectomies were performed using a colpotomy approach modified slightly from that previously described (Singh *et al.* 1998). Briefly, caudal epidural anesthesia was induced Lidocaine HCL 2% and Epinephrine USP (5ml; Bimeda-MTC Animal Health Inc., Lavaltrie, QC, Canada) at the sacro-coccygeal or first intercoccygeal space. The perineum was scrubbed using an iodine-based detergent and solution. A small incision in the cranial aspect of the vaginal fornix was made using a scalpel blade and the peritoneum was manually ruptured to allow access to the peritoneal cavity. Local anesthesia of ovarian pedicle was induced using a lidocaine-soaked gauze, and a plastic clip was placed in the mesovarium to compress the ovarian vessels. The chain of an ecraseur (19" Chassaignac; German-made; Jorgensen Lab, Colorado, USA) was looped around the ovary and slowly tightened until the ovary was excised. The ovaries were placed in polyethylene bags, kept in ice, and transported to the laboratory within 5 minutes after collection. The number of follicles was counted and the three largest follicles were selected. The selections were based on previous ultrasound-based sketches? and confirmed using a ruler to measure follicle diameter once the follicles were opened. The goal was to collect antral and mural granulosa cells. Antral granulosa cells are the cells that are easily found free in the follicular fluid, while the mural granulosa cells surround the inner wall of the follicle. To collect the antral granulosa cells, selected follicles were aspirated using a 20 gauge needle and a syringe. Each follicle was flushed 3 times with Delbecco's phosphate buffer saline (dPBS, Invitrogen Corporation, catalog 14190-144, Burlington, ON, Canada). The cumulus-oocyte-complex (COC) was then searched for and separated from follicular aspirates. Undiluted follicular aspirates were centrifuged to separate the follicular fluid from antral granulosa cells (pellet). The now-collapsed follicles were sliced in half using a scalpel blade and measured. The inside of the follicular wall was scraped with a microbiology culture-loop (LightLabs, cat#PD104, Dallas, USA) to remove the mural granulosa cells, which were placed together with the

antral granulosa cells from the aspirate . So the complete granulosa sample (mural and antral) were then snap-frozen in liquid nitrogen and kept at -80^{o}C for microarray and RT-PCR analyses.

6.3.3 RNA extraction and amplification

Total RNA was extracted using Trizol extraction method according to the manufacture's instruction (Invitrogen Life Technology) and resuspended in 50µl of nuclease-free water. RNA was purified using the Arcturus *PicoPure RNA* Isolation and purification Kit (Catalog KIT0204 Applied Biosystem Ontario, Canada) following manufacture's protocol. The purification process includes DNAse treatment to remove genomic DNA and final purified RNA was recovered in 15µl of elution buffer. RNA quality was evaluated using Bioanalyzer-2100 (Agilent Technologies, Palo Alto, CA, USA) with the RNA NanoLab Chip (Catalog # 5067-1511, Agilent Technologies). RNA samples with RNA integrity number (RIN) greater than 5 were used for microarray hybridizations. A linear amplification process was chosen with the intent of increasing the amount of genetic material required for microarray hybridizations. Equal amounts of RNA from the three largest follicles were pooled and a total of five nanogram RNA from the pooled sample was used for RNA amplification. Linear amplification was performed using two 6-hour rounds of T7 RNA polymerase (RiboAmp HS^{Plus} RNA Amplification Kit; Molecular Devices, Sunnyvale, CA, USA) following manufacturer's directions and the produced antisense RNA (aRNA) amount was measured using NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA).

6.3.4 Sample Labeling, Hybridization and Microarray Scanning

For each sample, 2.5 µg of aRNA were labelled using DY-547/647 (Red - CY5 and Green CY3) fluorescent dyes from ULS Labelling Kit (EA-006, Kreatech Diagnostics, Amsterdam, The Netherlands) according to the manufacturer's protocol. With the intent of removing any non-reacted ULS-label material, another round of aRNA purification was performed using the Pico-Pure RNA Isolation Kit but without DNAse I treatment. Pure labeled aRNA was eluded in 11µl elution buffer. Labelling efficiency was

measured using the NanoDrop ND-1000. Minimum of 30 pmol/µg of labeling signal was required to proceed with hybridization. A hybridization mix was prepared using 825ng of each cyanine (Cy3 and Cy5) labeled amplified aRNA, agilent and tomato spikes, nuclease free water, 10X blocking agent and a 25X fragmentation buffer, in a total volume of 55µl, which was pipeted into the hybridization slides. Three biological replicates in each group (FSH starvation vs. control) were used in the experimental design, in a dye-swap set up. Overall, 6 hybridizations were performed using a custom-build bovine oligo array slide (EmbryoGENE EMBV3 manufactured by Agilent; Design ID: 028298, GEO accession # GPL13226). The slide contained a total of 45220 number of oligo nucleotide probes. Each probe had a duplicate and the slide also included Agilent's positive and negative controls in 4x44K format. Oligo sequences were taken from Oligo Microarray Consortium database (BOMC, http://www.bovineoligo.org).

Hybridization were performed (Agilent Technologies Inc., Wilmington, DE, USA) using 2x GEx Hybridization Buffer HI-RPM, at 65^{o}C in preheated oven for 17 hours with rotator speed at 10 rpm. Slides were washed with two buffers from the Gene Expression (GE) wash buffer kit (Agilent technologies Inc. DE USA, catalogue # 5188-5327), according to manufacturer's protocol. Later, slides were dipped in 100% acetonitrile for 10 seconds at room temperature and washed with stabilization and drying solution for 30 seconds at room temperature.

The slides were immediately scanned and visualized using Power scanner^{TM} (Tecan US Inc, Durham, NC USA). After image acquisition, scanned images were analyzed and quantified using with Array-Pro Analyzer software (Media Cybernetics, Silver Spring, MD, USA).

6.3.5 Data normalization and Statistical Analysis

Raw signal intensity files were uploaded to EmbryoGENE Laboratory Management Information System (LIMS) and microarray Analysis platform (ELMA). Quality control was evaluated using Gydle software (http://www.gydle.com/). Signal intensity files were analyzed using the FlexArray software, version 1.6.1 (Blazejczyk *et al*. 2007), which is implemented with function from Bioconductor. Simple background

subtraction was done with the Flexarray software using the median foreground intensity files. If background intensity was higher than the obtained foreground intensity, negative values were replaced with 0.5 as default. Data was normalized within and between arrays, using Loess and Quantile normalization methodology, respectively. Limma simple was performed to obtain differentially expressed genes in FSH starvation group compared with control group (Smyth 2004, Smyth 2005), using fold change of 2 and P value of 0.05 as a threshold. False Discovery Rate (FDR) using Benjimeni-Hocheberg method was run to narrow down the true positive genes. A fold change of 2 and P value of 0.05 was also used for FDR.

6.3.6 Functional Annotation and Pathway analysis

The differentially expressed gene list, generated after Limma analysis, was uploaded into Ingenuity Pathways Analysis (IPA Version: 14400082; Ingenuity Systems, www.ingenuity.com) to identify gene networks. Gene networks were used to identify likely biological functions, molecular processes and disorders, and pathways most related to the gene list. IPA analyses are based on human and mouse studies.

6.3.7 Real-time PCR

Seven genes (LRP8, RGS2, PTGS2, PTX3, VNN1, INBHA and NTS) were selected for rtPCR. Primers were designed using Primer3 v.0.4.0 website (http://frodo.wi.mit.edu/primer3/); analyzed using IDT PrimerQuest tool - Oligo Analyzer website (http://scitools.idtdna.com/analyzer/Applications/OligoAnalyzer/) and blasted using NCBI (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome ; http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthome. Selected primers met the following criteria: 20 to 24 base pairs; 55º to 65ºC melting temperature; 40 to 60% of CG content; no hairpin, self-dimer or hetero-dimer formation, and specific to the gene of interest. A list of selected primers is presented in Table 6.1.

After selection, primers were tested by performing RT-PCR of cDNA from pooled granulosa cell samples. After the cDNA was amplified during the PCR reaction (see below), the amplicon was run by electrophoresis in a standard 1% agarose gel to determine the size of the band. The band was cut and eluted using a QIAquick Gel Extraction kit (Cat# 28704, Qiagen, Toronto, ON, Canada), quantified using NanoDrop ND-100 and sequenced (3 x ABI 3730xl Sanger sequencing). Primers were used only if the sequence matched the desired amplicon. The amplicon was also used for generating a standard curve. The standard curve went from 10^{-2} to 10^{-11} ng/µl.

Real time PCR was performed in a Stratagene Mx3005P fast thermal cycler (Applied Bioscience) using SYBR Green master mix (Applied Bioscience). Cycle threshold (Ct) were recorded; the expression of the interest gene was normalized to geometric mean of UBE2D2, EIF2B2 and SF3A1 using the Relative Expression Software Tool (REST 2009, Quiagen).

Table 6.1. Primers used for real-time PCR.

Genes	Strand	Primer sequence	Annealing Temperature (°C)
EIF2B2	Forward	5'-CATGAGATGGCAGTCAATTTGT-3'	53.6
	Reverse	5'-CTTGAACATAGGAGCACAGACG-3'	55.5
SF3A1	Forward	5'-TGTGTCCCTCTTGCTGAGTTT-3'	56.4
	Reverse	5'-ATTCCTGGTTTCACGTCTCCTA-3'	55.5
UBE2D2	Forward	5'-TGGACTCAGAAGTATGCGATGT-3'	55.8
	Reverse	5'-CTTCTCTGCTAGGAGGCAATGT-3'	56.6
NTS	Forward	5'-AGTGTTCCCTCTTGGAAAATGA-3'	60
	Reverse	5'-TCTTCCTGAATCAACTCCCAGT-3'	60.1
PTGS2	Forward	5'-AAAGCTCTAGGGGGTTCTCG-3'	56.3
	Reverse	5'-TGTCAGCACATCCAGGGTAA-3'	56
PTX3	Forward	5'-GGCAGACTCACAGGCTTCAATATC-3'	57.6
	Reverse	5'-CCTTCTCCAGTCTCCCTTTCAACT-3'	58.2
RGS2	Forward	5'-AAAGCCGCAGATCACCACAGAA-3'	59.3
	Reverse	5'-TCCAGCTTGAGACACACCACAT-3'	58.6

	Forward	5’- TATTCTCTTCCACGATCCTGCT -3’	58.6
VNN1	Reverse	5’- TTCCACTCCCTGTCATTTTCTT -3’	58
	Forward	5’-ACGCAAAGTTCTCGCAAGCTCA-3’	59.7
LRP8	Reverse	5’-TGCCATTTCCTCCTCAAACAGG-3’	57.6
	Forward	5’- CCAAAGGATGTACCCAACTCTC -3’	59.8
INHBA	Reverse	5’- GTCCGATGTCGTCCTCTATCTC -3’	60.7

6.3.8 Radio-immunoassays

Follicular fluid concentrations of estradiol and progesterone were measured using radioimmunoassay. Slaughterhouse ovaries were used to obtain a charcoal-extracted pool of follicular fluid, which was used to prepare the standards and dilute follicular fluid samples. The standard curve ranged from 5 to 1000 pg/ml for estradiol and 0.1 to 40 ng/ml for progesterone. Samples were diluted using the charcoal-extracted pooled follicular fluid so that hormone concentrations fell within the limits of the standard curve and samples were assayed in duplicates. Estradiol was measured with a modified human double-antibody RIA Kit (Catalog # KE2D1, Coat-A-Count; Siemens Healthcare Diagnostics Inc.; Mississauga, ON, Canada), dilutions ranged from 1:25 to 1:500. Progesterone was measured using commercial radioimmunoassay kit (Catalog # TKOP1, Coat-A-Count; Siemens Healthcare Diagnostics Inc.; Mississauga, ON, Canada) and all samples were diluted 1:10. All samples for progesterone analysis were analyzed in a single assay and the intra-assay coefficient of variation was 5.6%. Estradiol was measured in two different assays; the intra-assay coefficient of variation was 11% and the inter-assay coefficient of variation was 8.1%. Hormone data were compared between groups by analysis of variance using a general linear model procedure (GLM) (SAS Learning Edition 4.1; SAS Institute; Cary, NC, USA).

6.4 Results

6.4.1 Differential gene expression profile

There are a total of 671 differentially expressed genes in granulosa cells from the FSH starvation group compared with those from the reference group (fold change =2 and P value ≤ 0.05; Figure 6.1). Of these, 396 genes had significantly lower expression in the FSH starvation group (i.e. down-regulated genes), while 275 genes had significantly higher expression (i.e. upregulated genes). Ninety-four of these transcripts were novel (function are not known). Table 6.2 lists the 10 most up- and down-regulated granulosa cell gene transcripts after FSH starvation compared to the reference group.

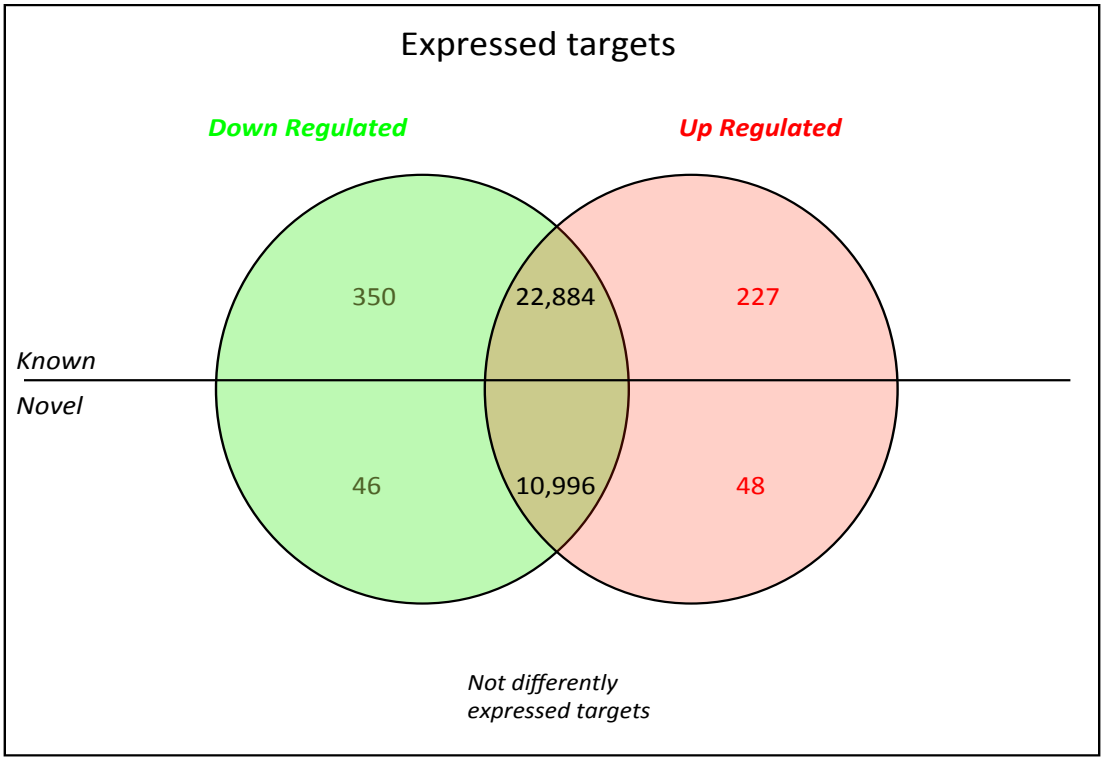


Figure 6.2. Venn diagram summarizing microarray analysis of bovine granulosa cells after ovarian superstimulation followed by a period of FSH starvation vs. no starvation. Up- and down-regulated gene were identified using fold-change of 2 and a P-value of ≥0.05.

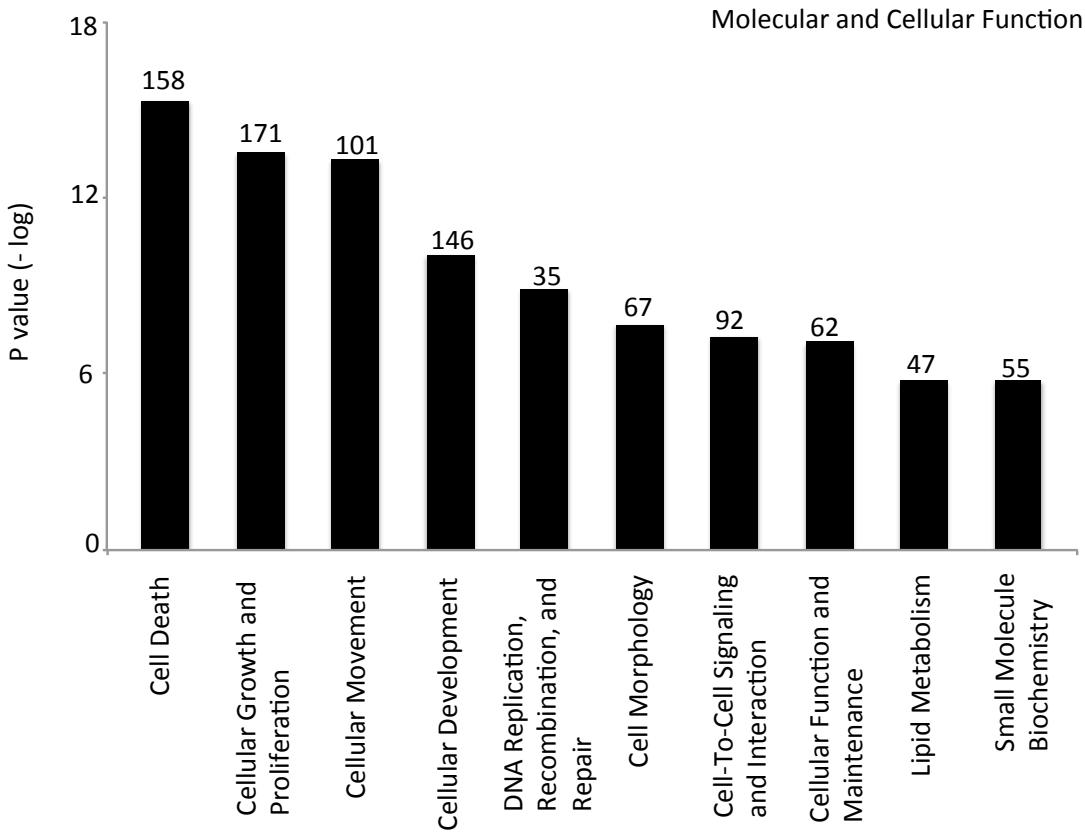
Table 6.2. Top 10 upregulated and down-regulated gene transcripts in bovine granulosa cells after ovarian superstimulation followed by a period of FSH starvation vs. no starvation.

Genes	Description	P Value	Fold change
UpRegulated			
VNN1	Vanin 1	$5.8x10^{-15}$	6.662
ANK3	Ankyrin 3	$3.6x10^{-12}$	3.315
ANGPT2	Angiopoietin 2	$5.6x10^{-10}$	3.314
TRIB2	Tibbles homolog 2 (Drosophila)	$5.8x10^{-06}$	3.123
INHBA	Inhibin beta A	$1.9x10^{-05}$	2.927
LRRC17	Leucine rich repeat containing 17	$4.3x10^{-10}$	2.902
LRP8	Low density lipoprotein receptor-related protein	$1.2x10^{-05}$	2.828
PDK4	Pyruvate dehydrogenase kinase isozyme 4	$2.2x10^{-07}$	2.799
GADD45A	Growth arrest and DNA damage-inducible, alpha	$6.1x10^{-07}$	2.772
APOD	Apolipoprotein D	$2.6x10^{-08}$	2.674
Down-Regulated			
NTS	Neurotensin	$9.8x10^{-08}$	-4.337
RGS2	Regulator of G protein signaling 2	$7.6x10^{-07}$	-4.045
POSTN	Periostin, Osteoblast specific factor Cyclin D2	$6.7x10^{-07}$	-3.860
PTGS2	Prostaglandin endoperoxide synthase 2	$5.8x10^{-07}$	-3.855
GRIA3	Glutamate receptor, ionotropic, AMPA3	$5.1x10^{-06}$	-3.698
PTX3	Pentraxin 3	$4.4x10^{-09}$	-3.688
CRISPLD2	Cysteine rich secretory protein LCCL domain containing 2	$3.9x10^{-05}$	-3.521
TM4SF1	Transmembrane 4 L six family 1	$2.9x10^{-08}$	-3.516
PLA2G4A	Phospholipase A2 group Iva (cytosolic, calcium dependent)	$1.7x10^{-06}$	-3.302
UBD	Ubiquitin D	$1.1x10^{-10}$	-3.203

6.4.2 Function, network and pathway analyses

The gene list obtained after limma analysis was up-loaded in Ingenuity, and function, network, and pathway analyses were performed. The top 10 functions and the top 10 pathways are shown in Figure 6.3 and 6.5. Network analysis identified down regulation of processes involving LH response and follicular maturity (Figure 6.4). Specifically, gene expression in granulosa cells from FSH-starved follicles reflected growth arrest (down regulation of genes related to cell proliferation) and immaturity (down regulation of genes related to ovulation).

A)



B)

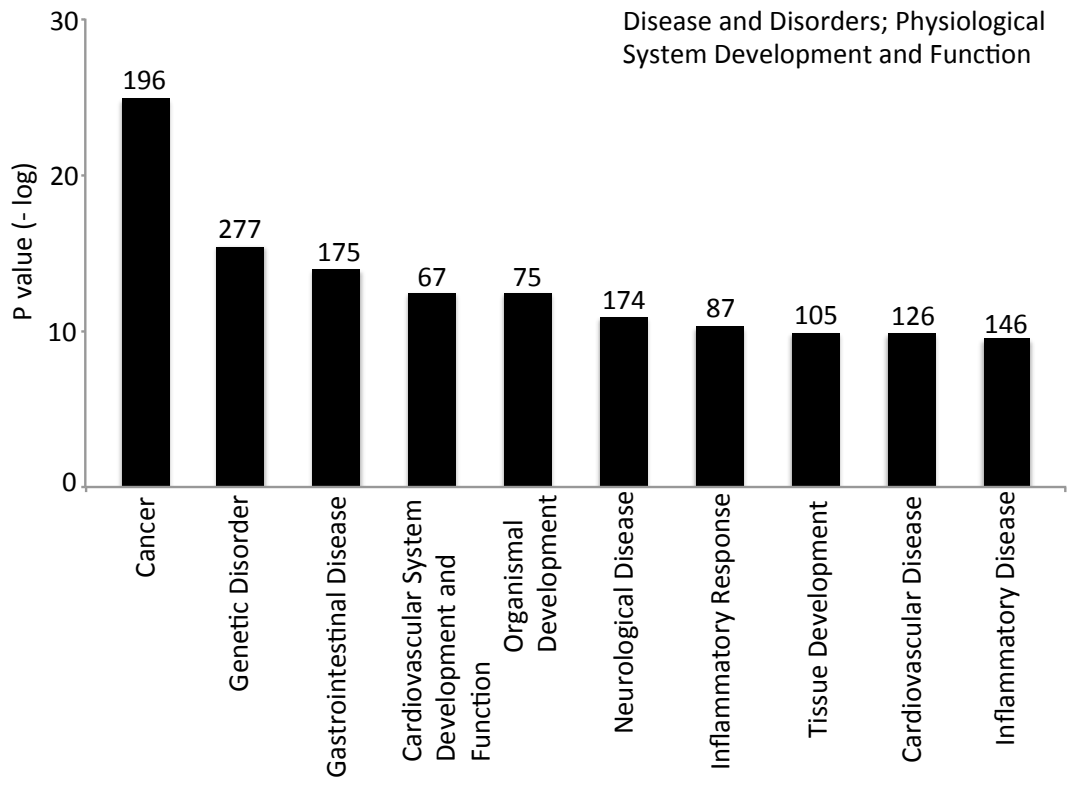


Figure 6.3. Function analysis of gene expression in bovine granulosa cells after ovarian superstimulation followed by a period of FSH starvation vs. no starvation. Taller bars represent a greater significance (log P-value) in the difference between groups. Numbers at the top of the bars indicate the number of genes involved with each function. The top 10 molecular and cellular functions (A) and the top 10 disease and disorders/ physiological functions (B) are illustrated.

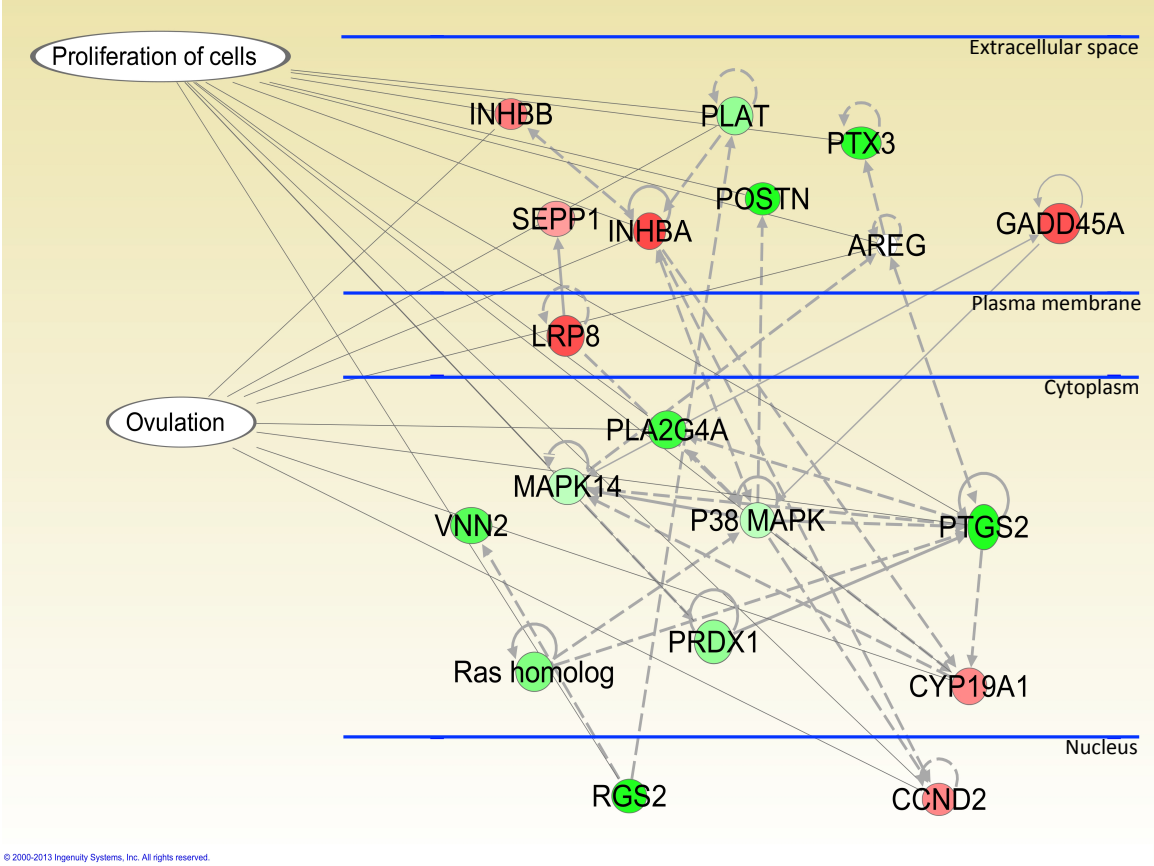
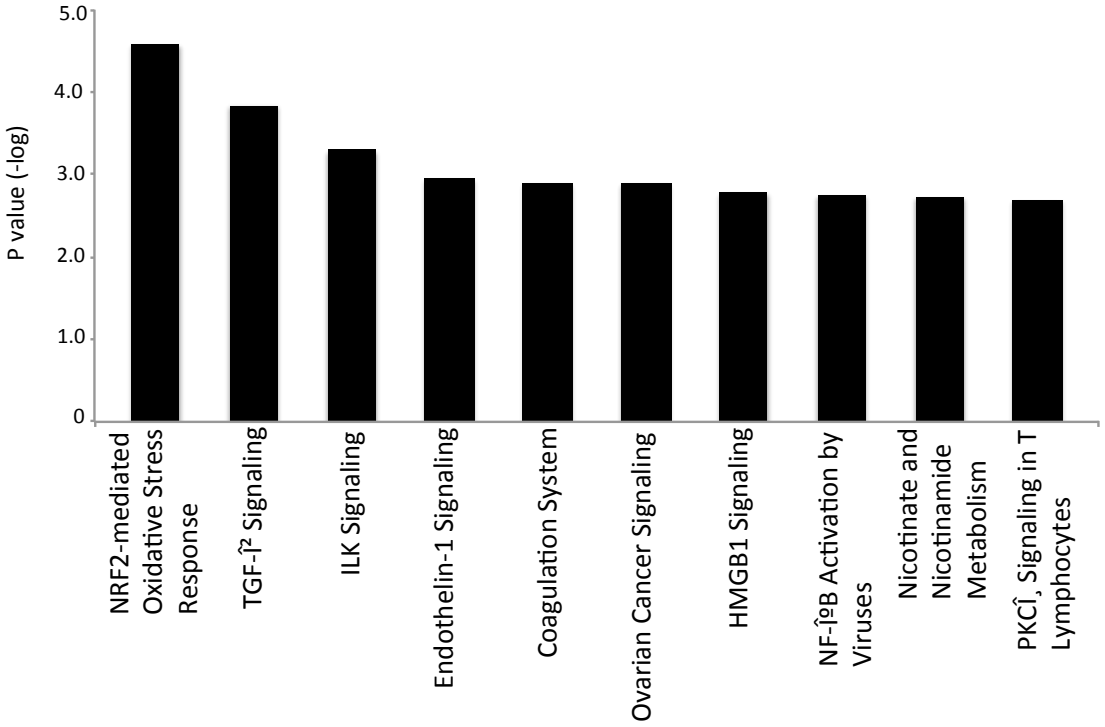


Figure 6.4. Network of upregulated (red) and down-regulated (green) mRNA transcripts in bovine granulosa cells after ovarian superstimulation and a period of FSH starvation vs. no starvation. Differences in color intensity of molecules show the degree of up- or down-regulation. The upregulated gene transcripts are: Cyclin D2 (CCND2), Cytochrome P450 family 19 subfamily A polypeptide 1 (CYP19A1), Low-desity lipoprotein receptor related protein 8 (LRP8), Inhibin beta B (INHBB), Inhibin beta A (INHBA) and Selenoprotein P plasm 1 (SEPP1), Growth arrest and DNA damage-inducible gene (GADD45A). The down-regulated gene transcripts are: Regulator of G-protein signalling 2 (RGS2), GTPase Rho (Ras (homolog)), Peroxiredoxin 1 (PRDX1), Prostaglandin endoperoxide synthase 2 (PTGS2), Vanin 2 (VNN2), Mitogen activated protein kinase P38 (P38 MAPK), Mitogen activated protein kinase 14 (MAPK14), Phospholipase A2 group IVA (PLA2G4A), Periostin osteoblast specific factor (POSTN), Pentraxin 3 (PTX3) and Plasminogen activator (PLAT).

A)


P value (-log)
5.0
4.0
3.0
2.0
1.0
0
NRF2-mediated Oxidative Stress Response
TGF-Î² Signaling
ILK Signaling
Endothelin-1 Signaling
Coagulation System
Ovarian Cancer Signaling
HMGB1 Signaling
NF-ÎºB Activation by Viruses
Nicotinate and Nicotinamide Metabolism
PKCÎ¸ Signaling in T Lymphocytes

B)

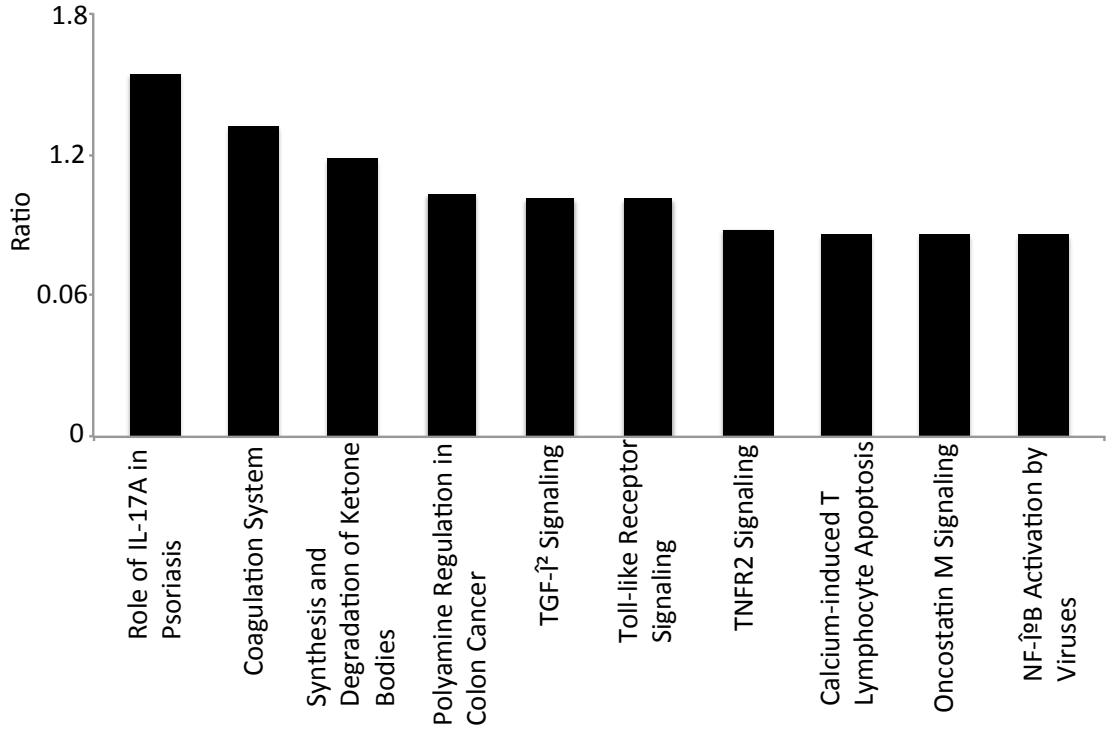


Figure 6.5. Canonical pathways analysis of gene expression of granulosa cells from superstimulated follicles after FSH starvation, using Ingenuity Pathway Analysis software (IPA). A) Top 10 pathways analysis based on -$\log_{10}$ p-value. B) Top 10 pathway analysis based on a score ratio. The score ratio was calculated by IPA and is the number of differentially expressed molecules in the gene list/ number of genes known to be involved in the pathway.

6.4.3 Real- Time PCR validation

Based on microarray data and functional analysis, a total of 7 genes (NTS, PTGS2, PTX3, RGS2, INHBA, VNN1 and LRP8) were selected for validation with RT-PCR. Those selected genes were quantified in three independent biological replicates from FSH starvation and control groups. Results showed positive validation of differential expression for 5 of the 7 genes (71%; NTS, PTGS2, PTX3, RGS2 and VNN1) using a 90% confidence level (Figure 6.4). Although two genes did not have a

statistical significance (LRP8 and INHBA), they followed the same trend as microarrays as shown in Figure 6.4.

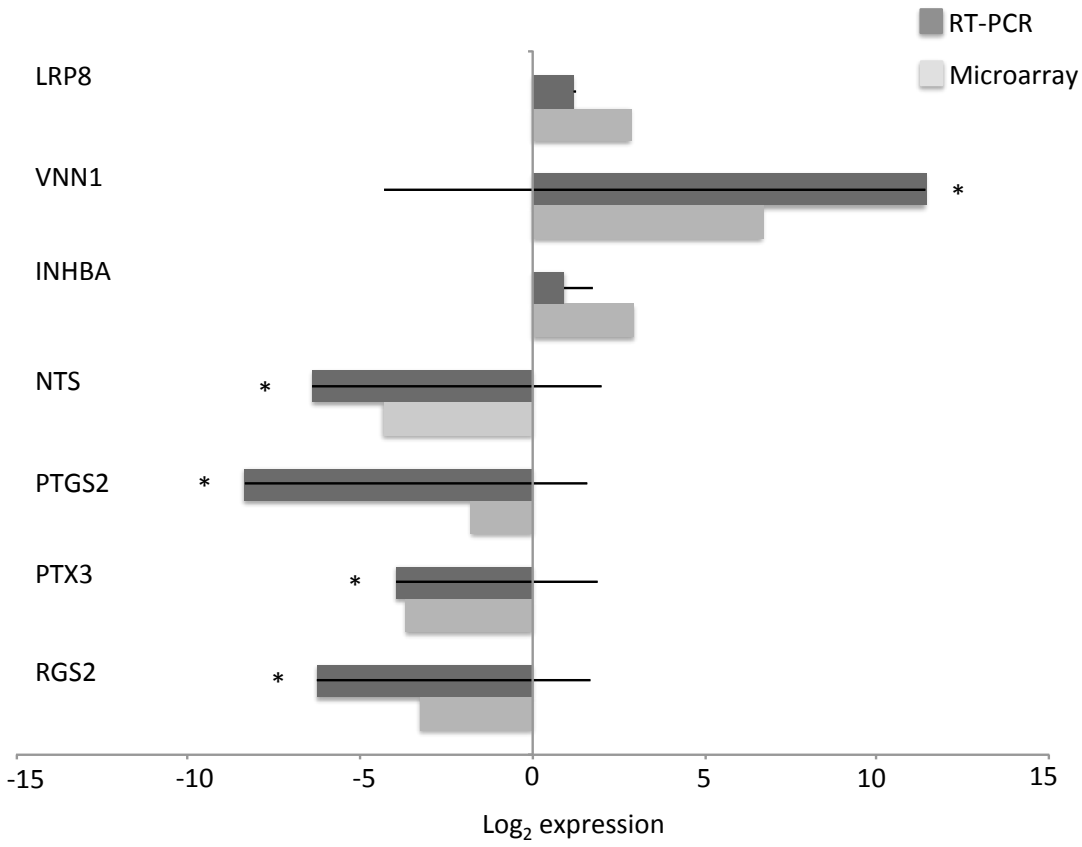


Figure 6.4. Relative quantification (log_2 fold change) of the mRNA levels in bovine granulosa cells form superstimulated follicles after FSH starvation compared with those from the continuous 7-day FSH treatment. RT-PCR data analysis was performed using REST 2009 program. Light grey bars represent expression of mRNA transcripts in microarray experiment while dark grey bars represent expression of the same transcript obtained by RT-PCR. Asterisk (*) above each bar represent statistical difference between FSH starvation in relation to the control in RT-PCR experiments (n=3 samples per group).

6.4.4 Follicular Fluid Analysis

No difference was detected in follicular fluid estradiol concentrations between the FSH starvation group and reference group (36.6 ± 23.2 and 21.3 ± 4.8 ng/ml; mean±SEM, respectively; P=0.53). Follicular fluid progesterone concentrations were lower in the FSH starvation group than in the reference group (212.9 ± 24.5 and 62.7 ± 18.0 ng/ml; respectively; P<0.001). Estradiol:progesterone ratio in the follicular fluid was not different between the FSH starvation and reference groups (0.61 ± 0.4 and 0.13 ± 0.03, respectively; P=0.2).

6.5 Discussion

Microarray-based comparison is a hypothesis generating tool for future evaluations In the present study microarray technology was used to examine the effect of withdrawal of FSH support (FSH starvation) during superstimulation protocol in cattle. Results of the present study provide rationale for four hypotheses: 1) FSH starvation results in growth arrest by decreasing expression of genes involved in cell proliferation; 2) FSH starvation alters genes involved in LH responsiveness and ovulation; 3) FSH starvation leads to ovulation failure by disturbing expression of genes in the prostaglandin-signaling pathway; and 4) FSH starvation affects the expression of granulosa cell gene markers for oocyte quality and embryo development.

Prolongation of the follicular growing phase during superstimulation resulted in a greater superstimulatory response and more synchronous ovulations (Dias *et al.* 2012a, Garcia Guerra *et al.* 2012). Further, oocyte competence after 7 days versus a standard 4-days superstimulation protocol did not differ. Continuous FSH support during the extended protocol is crucial for ovulation success. When a total of 96 hours of FSH starvation was performed after initial 4 Day FSH treatment, 50% of follicles failed to ovulate (Dias *et al.* 2012a), whereas 144 hours of FSH starvation under low progesterone environment resulted in 100% ovulation failure (Jaiswal R.S. *et al.* 2006). Authors suggested that both 96 and 144 hours of FSH starvation during superstimulation resulted

in either atretic or persistent follicles; however, oocyte competence could not be accessed in those studies due to the lack of ovulation. Therefore, in a similar study (Chapter 3) follicular aspiration was performed to assess the effect of FSH starvation during superstimulation in oocyte competence after in vitro fertilization. Cumulus oocyte complexes from the FSH starvation group were poor quality (grade 3 and 4) and partially expanded, while in the group with continuous FSH support COC were good quality (grade 1 and 2) and fully expanded. The developmental competence of the oocytes collected from the FSH starvation group was severely compromised; 96% failed to fertilize and those that developed into embryos did so more slowly than normal (Chapter 3). In a different study (Dadarwal 2012) oocytes after 96 hours of FSH starvation accumulated more lipid droplets in the ooplasm and failed to re-initiate meiosis compared to oocytes after a long FSH treatment. Therefore, based on previous studies (Jaiswal R.S. *et al.* 2006, Dias *et al.* 2012a) 84 to 144 hours of FSH starvation during superstimulation leads to loss ovulatory capability, fertilization failure, nuclear maturation failure, and decreased oocyte competence. In the present study, the analysis of gene expression of granulosa cells that underwent FSH starvation during superstimulation helps to understand the molecular changes that led to the loss of follicular and oocyte function.

The network analysis (Figure 6.3) pointed out 12 genes involved in cell proliferation, 8 of which were down-regulated (PLAT, PTX3, POSTN, PLA2G4A, PTGS2, PRDX1, MAPK14 and RGS2) and 4 were upregulated (CCND2, CYP19A1, INHBA and INHBB). Based on the down-regulation of many genes involved in cell proliferation, we postulate that FSH starvation caused growth-arrest of granulosa cells. The growth-arrest and DNA damage-inducible gene (GADD45A) was one of the top 10 upregulated genes suggesting that FSH starvation may have created a stressful environment in the granulosa cells. GADD45A is one component of the p53 pathway and is part of a response mechanism from exogenous damage agents (Smith *et al.* 1994). It is activated after DNA damage and is a marker of atresia (Hayashi *et al.* 2010). However, analysis of estradiol and progesterone from the follicular fluid showed equally high levels of estradiol and estradiol:progesterone ratio in both FSH starvation and control group,

indicating that granulosa cells are still steroidogenically active and may not have entered the final stages of atresia.

Neurotensin (NTS) is a secreted tridecapeptide (13 amino acid protein), which is widely distributed throughout the central nervous system (Carraway & Leeman 1973); however little information about its role in ovarian function is available. Neurotensin was first isolated from extracts of bovine hypothalamus and is implicated in the regulation of LH release (Carraway & Leeman 1973). It was demonstrated that estradiol increases expression of rat NTS mRNA in frontal cortex (medial preoptic nucleus from hypothalamus) from middle-aged rat missing ovaries (Sarvari *et al.* 2010) implicating its role in the regulation of hypothalamic-pituitary-gonadal axis. Follicle formation was delayed in NTS receptor null mice (Kerr *et al.* 2009) and both the number of secondary follicles and FSH receptor (FSHR) expression were diminished. Transient exposure of wild-type ovaries to NTS increases FSH-receptor gene expression and Cyclin D2 levels (Kerr *et al.* 2009) – the later protein mediates FSH action. Therefore, NTS receptors seem to facilitate follicle development by inducing functional FSHR receptor. The fact that NTS is down-regulated after FSH starvation suggest that FSH also play a role in regulating this gene, perhaps trough a feedback mechanism. However, the expression of FSHR is not affected by FSH starvation in the present study.

Granulosa cells and oocyte need to be able to respond properly to LH surge to coordinate meiosis resumption and complete ovulation. Many genes are influenced by LH surge; those genes are refered as markers of LH responsiveness (Gilbert *et al.* 2011).. The network presented in this paper (Figure 6.3) summarizes some of the known markers of LH response and how FSH starvation is affecting their mRNA levles. Based on the present results many genes known to be upregulated by LH surge (RGS2, PRDX1, PTGS2, VNN2, MAPKP38, MAPK14, PLA2G4A, POSTN, PTX3 and PLAT) are down-regulated while markers of pre LH surge, CCND2, CYP19A1, INHBB, INHBA and SEPP1 (Ndiaye *et al.* 2005, Gilbert *et al.* 2011) are still upregulated in the FSH starvation group. As a result of exogenous exposure to LH, the later group of genes (markers of pre LH surge) were expected to be down-regulated (as happened in the reference group).

Therefore pre-ovulatory follicles from FSH starvation group failed to respond properly to LH surge. It is likely that these changes lead to failure of down-stream events in ovarian stroma (please see next paragaph). This could explain why those follicles fail to ovulate. Likewise, failure of proper granulosa cell-oocyte interaction after LH exposure may have caused decreased oocyte competence and failure to resume meiosis.

The changes in gene expression after an LH surge trigger many signalling pathways essential to ovulation process. Prostaglandins (PGs) produced within ovarian follicles in response to the ovulatory gonadotropin surge are essential for follicle rupture and oocyte release (Diouf *et al.* 2006). Phospholipase A2 group IV A (PLA2G4A) is one gene from the prostaglandin-signaling pathway, which is upregulated after LH surge (Duffy *et al.* 2005). PLA2G4A is involved in the release of arachidonic acid from membrane phospholipids (Takahashi *et al.* 2006). This gene is down-regulated after FSH starvation, which suggest delay of LH response and consequently disturbance in ovulation process. Another key member of the prostaglandin signalling is the prostagladin-endoperoxide synthase (PTGS2), also also known as cyclooxygenase 2 (COX2). PTGS2 converts arachidonic acid to prostaglandin (PGF), impacting PKA, MAPK, NF-B and PI3K/Akt pathways, directing cumulus cell expansion and survival (Takahashi *et al.* 2006). PTGS2 is down-regulated in FSH starvation group. Cyclooxygenase-2 (COX-2) derived prostaglandin E2 (PGE2) is a critical mediator of oocyte maturation and is a marker for ovulation process. Lack of expression of PTGS2 is associated with lack of ovulation (Shimada *et al.* 2006, Kurusu *et al.* 2009). Therefore, ovulation failure caused by FSH starvation during superstimulation protocol is probably due to disturbed expression of genes from the prostaglandin-signaling pathway.

The expression of the low-density lipoprotein receptor-related protein 8 (LRP8) is normally high in dominant follicles but decreases after LH surge (Agca *et al.* 2006, Fayad *et al.* 2007). Contrary to what it was expected, the LRP8 gene is within the top 10 upregulated gene list in this present study. Similarly, RGS2 is a marker of LH response and it is upregulated after LH surge. However, RGS2 is down-regulated in FSH starvation pre-ovulatory follicles. The increased expression of LRP8 and decreased

expression of RGS2 in the present study further strengthened our hypothesis that granulosa cells from FSH-starved follicles are not responding properly to LH surge. Similarly, pentraxin-3 (PTX3) is expected to be upregulated after LH surge (Varani *et al.* 2002, Agca *et al.* 2006); however in FSH starvation group PTX3 expression is down-regulated even after exogenous LH was given. PTX3 encodes C-reactive protein and serum amyloid P protein is essential for hyaluronan organization within the extracellular matrix of the cumulus oophorus, which is critical for oocyte fertilization and fertility. When ER stress was artificially created the expression of PTX3 is lowered (Wu *et al.* 2012). The decreased expression of PTX3 suggests that stressful conditions could be associated with FSH starvation. If Pentraxin-3 expression in COC are significantly reduced mitochondrial activity is also lowered, *in vitro* fertilization rates is reduced, and embryos is slower to develop to blastocysts (Zhang *et al.* 2005). Therefore, PTX3 plays a key role in the organization of the cumulus oophorus, extracellular matrix and in fertilization and is a marker of high fertility. Perhaps FSH starvation compromise oocyte competence by decreasing the expression of PTX3.

In conclusion, prolonged withdrawl of FSH during superstimulation decreased expression of genes related with cellular growth. Moreover, FSH starvation during superstimulation was associated with down-regulation of LH and ovulation markers, and of genes from the prostaglandin-signaling pathway, showing a disturbed response to LH leading to ovulation failure. FSH starvation leads to poor oocyte quality and retarded embryonic development since the gene markers of oocyte quality and embryo development are also decreased after FSH starvation.

Acknowledgements

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CHAPTER 7

GENERAL DISCUSSION

Several experiments were conducted to test the effects of follicular aging and duration of superstimulation on oocyte competence and granulosa cell gene expression in cattle. We specifically examined: 1) the effects of the duration of the growing phase of the ovulatory follicle (analogous to 3- or 2-wave cycle) and a total period of 96 h of FSH starvation on oocyte competence using a superstimulation model and *in vitro* fertilization (Chapter 3); 2) the effect of superstimulation treatment on major molecular and cellular pathways, as evidenced by gene expression of granulosa cells (Chapter 4); 3) the transcriptome profile of granulosa cells exposed to a short or long duration of the growing phase of dominant follicle during superstimulation (Chapter 5); and 4) the effect of FSH starvation on gene expression of granulosa cells (Chapter 6).

Based on the present studies, the main results were: 1) extending the period of FSH treatment during superstimulation resulted in prolongation of follicular growth, a greater number of large follicles available for oocyte collection and in 2.5 times more transferable embryos per heifer. A total of 96 h of FSH starvation (84 h of gonadotropin free + 12 h of LH) was associated with collection of low quality oocytes with severely retarded fertilization potential and developmental competence (Chapter 3); 2) compared to the granulosa cells of single (natural) dominant follicles, superstimulation treatment results in granulosa cells that lag behind in maturation and differentiation and do not respond properly to LH stimulus (Chapter 4); 3) extending superstimulation protocol by 3 days allows more time for pre ovulatory follicles to leave the growing stage and properly respond to LH stimulus, activating markers for ovulation and oocyte competence (Chapter 5); 4) the continuous support with FSH during the extended superstimulated protocol is crucial since FSH starvation disturbs genes involved in LH response, ovulation and also affects oocyte competence by disturbing gene markers of oocyte quality and embryo development (Chapter 6).

In the decade of the 60's a study involving gross and histological examination of ovaries at random stages of estrous cycle was the first to determine that follicles grow in a wave like pattern (Rajakoski 1960). At that time it was proposed that the estrous cycle was composed of 2 follicular waves (Rajakoski 1960). The confirmation of the 2-wave hypothesis came later with the counting and measurement of all follicles present at the ovaries during 14 (Pierson & Ginther 1986) and then 58 (Pierson & Ginther 1987) interovulatory intervals. The advent of ultrasonography allowed the continuous monitoring of the estrous cycle, which permitted the detection of either 2 or 3 follicular waves during one interovulatory interval (Pierson & Ginther 1988, Savio *et al.* 1988, Sirois & Fortune 1988, Knopf *et al.* 1989). However, the factors that determine the number of waves during the estrous cycle are still not fully known.

During the first wave of the estrous cycle, the dominant follicle undergoes a growing, static and regression stage, while during the ovulatory wave instead of regression the dominant follicle ovulates (Ginther *et al.* 1989a). Many reports suggest that ovulatory follicles from 2 or 3 wave cycles may differ regarding oocyte competence and fertility (Ahmad *et al.* 1997, Townson *et al.* 2002, Bleach *et al.* 2004). The ovulatory follicle from a 2-wave cycle grows for 3 days longer under a high progesterone environment compared to ovulatory follicle from a 3-wave cycle (Mihm *et al.* 2002, Noseir 2003). Thus, the ovulatory follicle from a 2-wave may contain an aged oocyte, with decreased fertility.

The effect of number of waves on fertility has been tested; however results contradict among studies (Ahmad *et al.* 1997, Townson *et al.* 2002, Bleach *et al.* 2004). Two studies (Ahmad *et al.* 1997, Bleach *et al.* 2004) suggested a higher fertility in 3-wave animals; however both studies failed to detect statistically significant differences in pregnancy rate. A third study (Townson *et al.* 2002) reported higher pregnancy rate in 3-wave animals compared to 2-wave animals; however, the P value in that study was 0.058 (n=106 animals). Those three studies (Ahmad *et al.* 1997, Townson *et al.* 2002, Bleach *et al.* 2004) were limited statistically, with a small number of animals. A much larger study (n=365) (Dias *et al.* 2012b) artificially created an environment during the ovulatory wave

analogous to a 2 or 3-wave cycle, but failed to detect differences in pregnancy rates. Therefore, it was concluded that an oocyte from a dominant follicle of the 2-wave cycle was equally fertile as the 3-wave one.

In two studies (Dias *et al.* 2012a, Garcia Guerra *et al.* 2012) superstimulation was also used to test the effect of a short or long duration of the growing phase of the ovulatory follicle (analogous to 3 and 2-wave cycles, respectively) on oocyte competence. Both studies reported statistically greater ovulatory response to the superstimulation model analogous to the 2-wave cycle and numerically higher number of transferable embryos; however no significant difference was found (Dias *et al.* 2012a, Garcia Guerra *et al.* 2012). In chapter 3 of this thesis, a similar superstimulation protocol was used to compare the effect of a short and long duration (Short FSH and Long FSH) of the growing phase of the ovulatory follicle on oocyte competence after *in vitro* fertilization (IVF). The results from chapter 3 confirmed that the superstimulatory response when the ovulatory follicles grow 3 days longer under a high progesterone environment (Short FSH; analogous to a 2-wave cycle) is increased. Moreover, the follicle aspiration technique used in our study (Chapter 3) allowed a direct assessment of oocyte quality, morphology and embryonic development; however most of the endpoints were not statistically different between Long and Short FSH (2 or 3-waves). Oocyte competence (number of collected oocytes that developed into blastocyst with 9 days of culture) was equally good when using the superstimulated model of a 2-wave cycle or 3-wave cycle. However, the Long FSH (analogous to the 2-wave cycle) had statistically greater number of competent zygotes (number of cleaved embryos that developed into blastocyst with 9 days of culture). Therefore, there is enough evidence that the age effect from a 2-wave cycle is not detrimental to oocyte competence; on the contrary, extending the superstimulation protocol by 3 days (analogous to a the 2-wave cycle) increases the superstimulation response and may be a better option (Chapter 3) and (Dias *et al.* 2012a, Garcia Guerra *et al.* 2012).

Superstimulation has been widely used in research programs, since it can increase the number of experiment units (n) used. Chapter 3 is a good example where

superstimulation was used as a tool to increase the number of experiment units (oocyte and embryos) tested. Superstimulation was used in all the studies presented in this thesis (Chapter 3, 4, 5, and 6). However, the use of superstimulation during research programs requires caution so no assumptions to a natural dominant follicle are mistakenly made. The comparative differences between superstimulated follicles and oocytes to ones from a natural cycle are not deeply known. Thus, superstimulated follicles and oocytes may not be a real representative of follicles and oocytes from a natural cycle. In Chapter 4, we studied the effect of superstimulation treatment on gene expression of granulosa cells compared to the dominant follicle from a natural cycle. The superstimulation protocol selected in that study (Chapter 4) was a standard 4 day protocol; the same protocol used to represent the superstimulated model of a 3-wave animal in Chapter 3. To access molecular profile of granulosa cells microarray technique was used in Chapters 4, 5 and 6. The design of the microarray experiments used in the present studies allow straight comparison of a group with a reference group; which mean that the upregulated genes in the tested group are consequently down-regulated in the reference group and vice versa. It is important to note that microarray technique is a hypothesis generator tool. Further confirmations of the hypotheses pointed by the microarray data analysis are required.

Superstimulation response is variable among individuals (Looney 1986, Mapletoft *et al.* 2002, Mapletoft & Bo 2011). Moreover, the number of follicles in superstimulated ovaries hardly reflects the number of superovulation obtained (Desaulniers *et al.* 1995). The reason for persistent anovulatory follicles after superstimulation may be attributed to the effects of superstimulation treatment on follicular cells (Desaulniers *et al.* 1995). It is suggested that superstimulated follicles from a standard 4 day protocol are not always matured enough to respond to the LH surge (D'Occhio *et al.* 1997a). The combined estradiol from all follicles of the superstimulated pool induce an earlier endogenous LH surge after withdrawal of progestagen (Roberge *et al.* 1995). Thus, the immature follicles from a conventional superstimulated protocol are being exposed to an earlier LH surge, which may lead to anovulation.

The superstimulation protocol used in Chapter 4, 5 and 6 included an exogenous administration of LH 24 hours after the CIDR removal. Therefore, markers of LH response were expected to be already upregulated (Gilbert *et al.* 2011, Barros *et al.* 2012, Gilbert *et al.* 2012). However, the genetic profile of the granulosa cells pointed to only a few markers of LH surge being upregulated after a 4 day superstimulation protocol (Chapter 4). This confirms the suggestion that follicles that undergo a standard 4 day superstimulation protocol are not always matured enough to respond to the LH surge (D'Occhio *et al.* 1997a). Most of the highly significant differently expressed genes from our gene list in that study (Chapter 4) were associated with matrix remodelling during the growing stage, which led to the hypothesis that granulosa cells collected after a standard 4 day superstimulation protocol lag behind in maturation and differentiation and do not respond properly to LH surge (Figure 4, Chapter 4). Interestingly, granulosa cells that underwent a 7 day extended superstimulation protocol had more markers of LH being upregulated compared to the standard 4 day protocol (Chapter 5). At least five markers of the 22 hr post exogenous LH application matches the upregulated gene list of that study (VNN, POSTN, PLA2G4A, GTPase, Cysteine); while most of the downregulated genes obtained are markers of pre LH surge or are known to be downregulated after LH surge (Chapter 5; CYP19A1, LRP8, CJA1, INHBA and SERPINE2). Perhaps the prolonged growing phase under superstimulation stimulus allowed more time for follicles to leave the growing stage, mature and properly respond to the LH surge. An unpublished study from our lab (Dadarwal et al.) evaluated the nuclear maturation of oocytes after a short 4-day versus a long 7-day superstimulation protocol. The long 7-day protocol obtained 3 times more oocytes being in Metaphase II stage compared to oocytes from a short 4-day protocol. Normally, oocytes reach the Metaphase II around 19-20 hours after the LH surge (Hyttel *et al.* 1989). Moreover, a different report showed that mitochondria in oocytes from a long 7 day superstimulation protocol were more active and the oocytes had a higher ATP content than oocytes from a short 4-day protocol. Higher ATP content is positively associated with oocyte maturation rates and healthy embryo development (Stojkovic *et al.* 2001). This information confirmed the hypothesis generated by my study (Chapter 5) that follicles from the Long FSH protocol are more mature and respond better to the LH surge.

The time of the LH administration during superstimulation protocol has shown to affect the effectiveness of the protocol (D'Occhio *et al.* 1999, Barros & Nogueira 2001, Baruselli *et al.* 2006, Barros *et al.* 2012). Increased number of viable embryos are obtained after superstimulation in zebu animals when a CIDR is left in place for 36 hours after PGF and the exogenous LH is given 12 hours after CIDR removal (48 hours after PGF) (Nogueira & Barros 2003). So, the LH surge is delayed by 48 hours in this protocol (named P36/48h). The same protocol was tested in *Bos taurus* animals; however the number of viable embryos was reduced (revised in (Baruselli *et al.* 2006)). Comparable number of viable embryos was only obtained in *Bos taurus* when LH was further delayed to 60 hours after PGF2α (P36/60hr) (Baruselli *et al.* 2006, Chesta *et al.* 2007). Moreover, the use of a GnRH agonist to suppress earlier endogenous LH surge during superstimulation has been tested (D'Occhio *et al.* 1997b). It was found that delaying the exposure to LH by 12 hours numerically increased the number of follicles that ovulated; however the study lacked statistical power to obtain a significant difference (D'Occhio *et al.* 1997b). Addionally, the study found the further delay in LH surge of 24 hours resulted in decreased number of transferable embryos (D'Occhio *et al.* 1997b). In the herein studies LH was given 12 hours after CIDR removal in all groups; however in Long FSH and FSH starvation the LH surge was delayed in relation to the wave (and in comparison to the Short FSH group) by extending the protocol by 72 hours (3 days). The main difference between the protocols described above and the Long FSH tested herein is the continuous FSH support. Where in the protocols described above the LH surge is delayed either by the use of a GnRH antagonist or by sub luteal levels of progesterone, without continuous FSH support, which is a similar idea to the FSH starvation group; however the duration of the FSH starvation differs. In the Long FSH group 6 extra doses of FSH were given, and the continuous support with FSH during the extended superstimulation protocol showed to be crucial to the success of the protocol.

Interestingly, the continuous FSH support during the extended superstimulation protocol is also important for ovulation success (Jaiswal R.S. *et al.* 2006, Dias *et al.* 2012a). A total of 144 hours of FSH starvation under a low progesterone environment

resulted in 100% ovulation failure (Jaiswal R.S. *et al.* 2006). When the total period of FSH starvation is reduced to 96 hours under a high progesterone environment 50% of the follicles failed to ovulate (Dias *et al.* 2012a). Authors from those two studies (Jaiswal R.S. *et al.* 2006, Dias *et al.* 2012a) suggested that both 96 and 144 hours of FSH starvation during superstimulation could possibly result in either atretic or persistent follicles; however oocyte competence could not be assessed in those studies due to the lack of ovulation. The results from the gene expression analysis after FSH withdrawal during superstimulation (Chapter 6) helped to understand this negative effect in ovulation. Granulosa cells after FSH starvation do not respond to LH surge since the markers of LH surge are turned off in that group (Chapter 6). Moreover, key genes during the ovulation process are also not being expressed (PTGS2, PLA2G4A and MAPK group; Chapter 6). Therefore, the lack of ovulation obtained in earlier studies is now confirmed at the molecular level.

In Chapter 3, ovum pick-up (OPU) was performed to bypass the lack of ovulation and be able to assess the effect of FSH starvation during superstimulation on oocyte competence after IVF. Cumulus cells oocyte complex (COC) from the FSH starvation group were poor quality and partially expanded, while COCs in the group with continuous FSH support were good quality and fully expanded (Chapter 3). There was a 96.4% of fertilization failure after FSH starvation and the few fertilized oocytes had a disturbed slower development (Chapter 3). Gene expression data after FSH starvation confirmed that gene markers of oocyte quality and embryo development (e.g. PTX3) are downregulated; the possible cause of the poor results observed in Chapter 3.

Previous studies from our laboratory (Dadarwal et. al; unpublished data) evaluated the effect of FSH starvation on oocyte maturation and organelle distribution in the oocyte cytoplasm. That study reported that 90% of oocytes from the FSH starvation group were unable to reach Metaphase II stage after 20 hours post LH and they were unable to progress beyond germinal vesicle stage, which indicates failure to resume meiosis (Dadarwal et. al; unpublished data). Moreover, oocytes from 96 hours of FSH starvation had more accumulation of bigger lipid droplets in the ooplasm but with less

ATP content than a long 7-day superstimulation protocol (Dadarwal et. al; unpublished data). Stored lipids are utilized by oocytes for the ATP generation needed during nuclear maturation (Downs *et al.* 2009, Dunning *et al.* 2010). It is speculated that in starved oocytes maturation is not occurring, which led to accumulation of bigger lipid content and less ATP (unpublished). The lack of maturation in staved oocytes is probably due to the disturbed response to LH surge. The genome analysis after FSH starvation (Chapter 6) showed many genes known to be upregulated by LH surge (RGS2, PRDX1, PTGS2, VNN2, MAPKP38, MAPK14, PLA2G4A, POSTN, PTX3 and PLAT) to be downregulated, while markers of pre LH surge (CCND2; CYP19A1; INHBB; INHBA and SEPP1) to be upregulated in the FSH starvation group. Therefore pre-ovulatory follicles from the FSH starvation group are not responding properly to LH surge. This could explain why those follicles fail to resume meiosis, fail to ovulate, fertilize and have decreased oocyte competence.

Growing or static follicles mature as they respond to LH surge and resume meiosis. Thus, the LH surge is a crucial event that delineates a change in the follicular status, from the growing (or static) phase to the pre-ovulatory phase. Regression is another possible path for follicles that do not ovulate. The evaluation of granulosa gene expression profile (on Chapters 4, 5 and 6) allowed determining the follicular stage of development. Figure 1 summarizes the main microarray findings (Chapters 4, 5 and 6) and the herein discussion. The main hypothesis pointed by the microarray data in Chapter 4, where a short 4 day superstimulation protocol is compared with the dominant follicle of a natural cycle, is that follicles were at the growing stage even after exogenous LH was given (Figure 1). Extending the standard protocol by 3 days provided more time for follicles to leave the growing phase, mature and properly respond to LH. Therefore, follicles from a long 7 day superstimulation protocol are at the pre-ovulatory stage (Figure 1), which is the right stage of development. Follicles only reach the pre-ovulatory stage in the long 7 day protocol if FSH support is continued. The withdrawal of FSH during a long 7 day protocol is detrimental to follicular health and results in growth arrest (Figure 1). The latest hypothesis is confirmed by cytoplasmic evaluation of oocytes, which reported cytoplasmic degenerative changes after 96 h of FSH starvation indicating atresia.

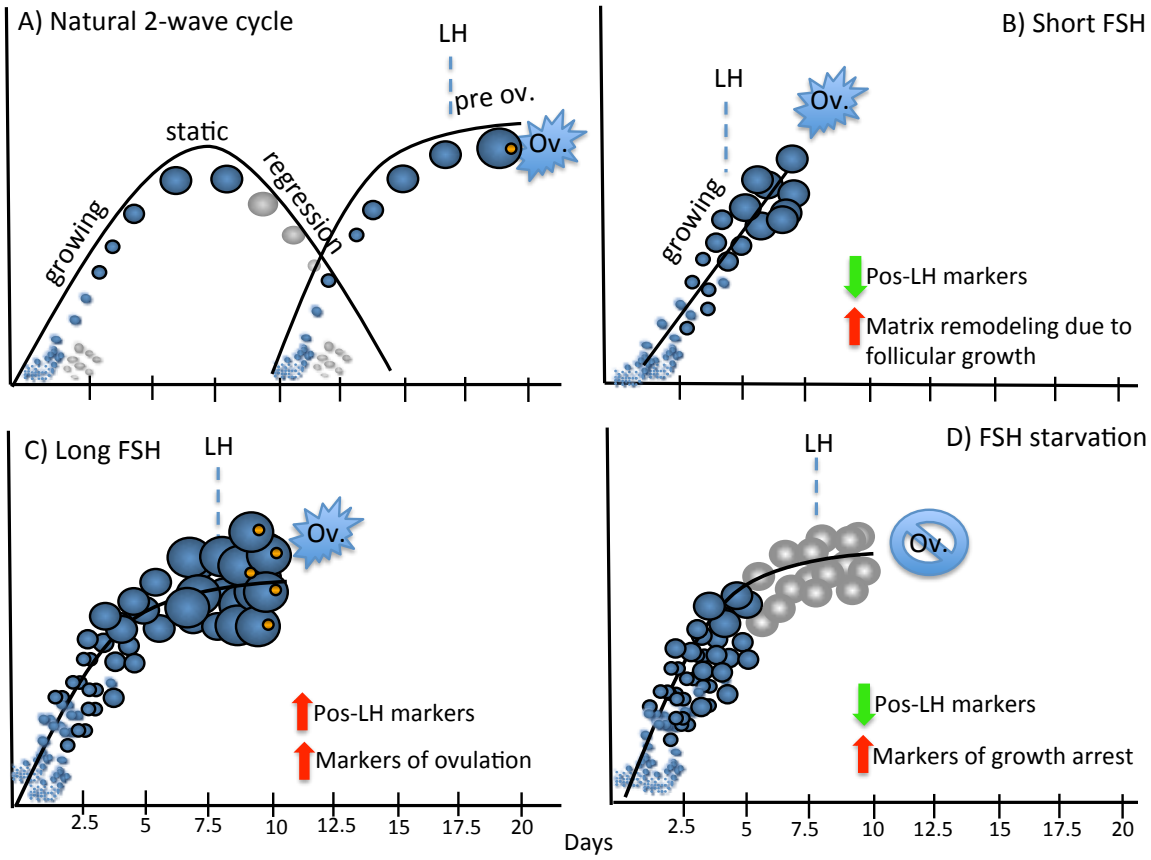

Figure 7.1. Diagram representing: A) follicular waves from a natural cycle; B) follicular growth after a short 4-day superstimulation (Short FSH); C) follicular growth after a long 7-day superstimulation (Long FSH), and D) follicular growth after a period of FSH starvation during a 7-day superstimulation protocol.

CHAPTER 8

GENERAL CONCLUSION

Based on the results presented in Chapters 3 to 6, we conclude that:

- Extending the period of FSH treatment during superstimulation results in prolongation of follicular growth, a greater number of large follicles available for oocyte collection and in 2.5 times more transferable embryos per heifer;
- Follicular maturation resulting from an extended period of FSH support is associated with collection of more COC that were fully expanded; however, numbers were insufficient to adequately test the hypothesis of an effect in developmental competence;
- A total of 96 h of FSH starvation (84 h of gonadotropin free + 12 h of LH) is associated with collection of low quality oocytes with severely retarded fertililization potential and developmental competence;
- Compared to the granulosa cells of single (natural) dominant follicles, superstimulation treatment results in granulosa cells that lag behind in maturation and differentiation;
- Extending superstimulation protocol by 3 days allow more time for pre ovulatory follicle to leave the growing stage and properly respond to LH stimulus;
- The extended protocol also activates markers for ovulation and oocyte competence;
- The continuous support with FSH during the extended superstimulated protocol is crucial since FSH starvation disturb genes involved in LH response and ovulation and affect oocyte competence by disturbing gene markers of oocyte quality and embryo development.

Therefore, the general hypotheses that the follicular aging and different durations of superstimulation alter granulosa gene expression and affect oocyte competence are supported.

CHAPTER 9

REFERENCES

Adams GP 1994 Control of ovarian follicular wave dymanics in cattle: implications for synchronization and superstimulation. *Theriogenology* **41** 19-24.

Adams GP. 1998. Control of ovarian follicular wave dynamics in mature and prepubertal cattle for synchronization & superstimulation. In *XX Congress of the World Association for Buiatrics*, pp. 595-605.

Adams GP 1999 Comparative patterns of follicle development and selection in ruminants. *Journal of reproduction and fertility. Supplement* **54** 17-32.

Adams GP, Jaiswal R, Singh J & Malhi P 2008 Progress in understanding ovarian follicular dynamics in cattle. *Theriogenology* **69** 72-80.

Adams GP, Kot K, Smith CA & Ginther OJ 1993a Effect of the dominant follicle on regression of its subordinates in heifers. *Canadian journal of animal science* **73** 267-275.

Adams GP, Kot K, Smith CA & Ginther OJ 1993b Selection of a dominant follicle and suppression of follicular growth in heifers. *Animal reproduction science* **30** 259-271.

Adams GP, Matteri RL & Ginther OJ 1992a Effect of progesterone on ovarian follicles, emergence of follicular waves and circulating follicle-stimulating hormone in heifers. *Journal of reproduction and fertility* **96** 627-640.

Adams GP, Matteri RL, Kastelic JP, Ko JC & Ginther OJ 1992b Association between surges of follicle-stimulating hormone and the emergence of follicular waves in heifers. *Journal of reproduction and fertility* **94** 177-188.

Adams GP, Nasser LF, Bo GA, Garcia A, Del Campo MR & Mapletoft RJ 1994 Superovulatory response of ovarian follicles of Wave 1 versus Wave 2 in heifers. *Theriogenology* **42** 1103-1113.

Adriaens I, Cortvrindt R & Smitz J 2004 Differential FSH exposure in preantral follicle culture has marked effects on folliculogenesis and oocyte developmental competence. *Human reproduction* **19** 398-408.

Agca C, Ries JE, Kolath SJ, Kim JH, Forrester LJ, Antoniou E, Whitworth KM, Mathialagan N, Springer GK, Prather RS & Lucy MC 2006 Luteinization of porcine preovulatory follicles leads to systematic changes in follicular gene expression. *Reproduction* **132** 133-145.

Ahmad N, Townsend EC, Dailey RA & Inskeep EK 1997 Relationships of hormonal patterns and fertility to occurrence of two or three waves of ovarian follicles, before and after breeding, in beef cows and heifers. *Animal reproduction science* **49** 13-28.

Alberts B, Johnson A, Lewis J, Raff M, Roberts K & Walter P 2002. The extracellular matrix of animals. In *Molecular Biology of the cell*.

Ali A & Sirard MA 2005 Protein kinases influence bovine oocyte competence during short-term treatment with recombinant human follicle stimulating hormone. *Reproduction* **130** 303-310.

Arici A, Oral E, Bukulmez O, Buradagunta S, Engin O & Olive DL 1996 Interleukin-8 expression and modulation in human preovulatory follicles and ovarian cells. *Endocrinology* **137** 3762-3769.

Assey RJ, Hyttel P, Greve T & Purwantara B 1994 Oocyte morphology in dominant and subordinate follicles. *Molecular reproduction and development* **37** 335-344.

Baenziger JU & Green ED 1988 Pituitary glycoprotein hormone oligosaccharides: structure, synthesis and function of the asparagine-linked oligosaccharides on lutropin, follitropin and thyrotropin. *Biochimica et biophysica acta* **947** 287-306.

Bao B & Garverick HA 1998 Expression of steroidogenic enzyme and gonadotropin receptor genes in bovine follicles during ovarian follicular waves: a review. *Journal of animal science* **76** 1903-1921.

Bao B, Garverick HA, Smith GW, Smith MF, Salfen BE & Youngquist RS 1997 Changes in messenger ribonucleic acid encoding luteinizing hormone receptor, cytochrome P450-side chain cleavage, and aromatase are associated with recruitment and selection of bovine ovarian follicles. *Biology of reproduction* **56** 1158-1168.

Barnes FL & Sirard MA 2000 Oocyte maturation. *Seminars in reproductive medicine* **18** 123-131.

Barros CM & Nogueira MF 2001 Embryo transfer in Bos indicus cattle. *Theriogenology* **56** 1483-1496.

Barros CM, Satrapa RA, Castilho AC, Fontes PK, Razza EM, Ereno RL & Nogueira MF 2012 Effect of superstimulatory treatments on the expression of genes related to ovulatory capacity, oocyte competence and embryo development in cattle. *Reproduction, fertility, and development* **25** 17-25.

Baruselli PS, de Sa Filho MF, Martins CM, Nasser LF, Nogueira MF, Barros CM & Bo GA 2006 Superovulation and embryo transfer in Bos indicus cattle. *Theriogenology* **65** 77-88.

Beg MA, Bergfelt DR, Kot K, Wiltbank MC & Ginther OJ 2001 Follicular-fluid factors and granulosa-cell gene expression associated with follicle deviation in cattle. *Biology of reproduction* **64** 432-441.

Benjamini Y & Hochberg Y 1995 Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society* **57** 289-300.

Berfelt DR, Lightfoot KC & Adams GP 1994 Ovarian synchronization following ultrasound-guided transvaginal follicle ablation in heifers. *Theriogenology* **42** 895-907.

Bergfelt DR, Lightfoot KC & GP. A 1994 Ovarian synchronization following ultrasound-guided follicular ablation in heifers. . *Theriogenology* **42** 895-907.

Berisha B, Steffl M, Welter H, Kliem H, Meyer HH, Schams D & Amselgruber W 2008 Effect of the luteinising hormone surge on regulation of vascular endothelial

growth factor and extracellular matrix-degrading proteinases and their inhibitors in bovine follicles. *Reproduction, fertility, and development* **20** 258-268.
Berkholtz CB, Lai BE, Woodruff TK & Shea LD 2006a Distribution of extracellular matrix proteins type I collagen, type IV collagen, fibronectin, and laminin in mouse folliculogenesis. *Histochemistry and cell biology* **126** 583-592.
Berkholtz CB, Shea LD & Woodruff TK 2006b Extracellular matrix functions in follicle maturation. *Seminars in reproductive medicine* **24** 262-269.
Bhoola KD, Figueroa CD & Worthy K 1992 Bioregulation of kinins: kallikreins, kininogens, and kininases. *Pharmacological reviews* **44** 1-80.
Bleach EC, Glencross RG & Knight PG 2004 Association between ovarian follicle development and pregnancy rates in dairy cows undergoing spontaneous oestrous cycles. *Reproduction* **127** 621-629.
Blondin P, Bousquet D, Twagiramungu H, Barnes F & Sirard MA 2002 Manipulation of follicular development to produce developmentally competent bovine oocytes. *Biology of reproduction* **66** 38-43.
Blondin P, Coenen K, Guilbault LA & Sirard MA 1997a In vitro production of bovine embryos: developmental competence is acquired before maturation. *Theriogenology* **47** 1061-1075.
Blondin P, Guilbault LA & Sirard MA 1997b The time interval between FSH-P administration and slaughter can influence the developmental competence of beef heifer oocytes. *Theriogenology* **48** 803-813.
Blondin P & Sirard MA 1995 Oocyte and follicular morphology as determining characteristics for developmental competence in bovine oocytes. *Molecular reproduction and development* **41** 54-62.
Bolstad BM, Irizarry RA, Anstrand M & Speed TP 2003 A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics* **19** 185-193.
Brackett BG & Oliphant G 1975 Capacitation of rabbit spermatozoa in vitro. *Biology of reproduction* **12** 260-274.
Brannstrom M, Bonello N, Wang LJ & Norman RJ 1995 Effects of tumour necrosis factor alpha (TNF alpha) on ovulation in the rat ovary. *Reproduction, fertility, and development* **7** 67-73.
Bridges PJ, Komar CM & Fortune JE 2006 Gonadotropin-induced expression of messenger ribonucleic acid for cyclooxygenase-2 and production of prostaglandins E and F2alpha in bovine preovulatory follicles are regulated by the progesterone receptor. *Endocrinology* **147** 4713-4722.
Carabatsos MJ, Sellitto C, Goodenough DA & Albertini DF 2000 Oocyte-granulosa cell heterologous gap junctions are required for the coordination of nuclear and cytoplasmic meiotic competence. *Developmental biology* **226** 167-179.
Carraway R & Leeman SE 1973 The isolation of a new hypotensive peptide, neurotensin, from bovine hypothalami. *The Journal of biological chemistry* **248** 6854-6861.
Cerutti PA 1989 Mechanisms of action of oxidant carcinogens. *Cancer detection and prevention* **14** 281-284.
Chesta P, Tribulo R, Tribulo H, Balla E, Baruselli PS & Bo GA 2007 Effect of the time of ovulation induction by gonadotropin-releasing hormone or pituitary

luteinizing hormone on ova/embryo production in superstimulated beff cows inseminated at a fixed time. *Reproduction, Fertility and Development* **19**.

Chohan KR & Hunter AG 2003 Meiotic competence of bovine fetal oocytes following in vitro maturation. *Animal reproduction science* **76** 43-51.

Christenson LK & Stouffer RL 1997 Follicle-stimulating hormone and luteinizing hormone/chorionic gonadotropin stimulation of vascular endothelial growth factor production by macaque granulosa cells from pre- and periovulatory follicles. *The Journal of clinical endocrinology and metabolism* **82** 2135-2142.

Chupin D, Combarnous Y & Procureur R 1984 Antagonistic effect of LH in commercially available gonadotrophins. **25** 167.

Clemens JW, Robker RL, Kraus WL, Katzenellenbogen BS & Richards JS 1998 Hormone induction of progesterone receptor (PR) messenger ribonucleic acid and activation of PR promoter regions in ovarian granulosa cells: evidence for a role of cyclic adenosine 3',5'-monophosphate but not estradiol. *Molecular endocrinology* **12** 1201-1214.

Clements JA, Mukhtar A, Holland AM, Ehrlich AR & Fuller PJ 1995 Kallikrein gene family expression in the rat ovary: localization to the granulosa cell. *Endocrinology* **136** 1137-1144.

Colman-Lerner A, Fischman ML, Lanuza GM, Bissell DM, Kornblihtt AR & Baranao JL 1999 Evidence for a role of the alternatively spliced ED-I sequence of fibronectin during ovarian follicular development. *Endocrinology* **140** 2541-2548.

Custer EE, Beal WE, Wilson SJ, Meadows AW, Berardinelli JG & Adair R 1994 Effect of melengestrol acetate (MGA) or progesterone-releasing intravaginal device (PRID) on follicular development, concentrations of estradiol-17 beta and progesterone, and luteinizing hormone release during an artificially lengthened bovine estrous cycle. *Journal of animal science* **72** 1282-1289.

D'Occhio MJ, Jillella D & Lindsey BR 1999 Factors that influence follicle recruitment, growth and ovulation during ovarian superstimulation in heifers: opportunities to increase ovulation rate and embryo recovery by delaying the exposure of follicles to LH. *Theriogenology* **51** 9-35.

D'Occhio MJ, Niasari-Naslaji A & Kinder JE 1997a Influence of varied progestogen treatments on ovarian follicle status and subsequent ovarian superstimulatory responses in cows. *Animal reproduction science* **45** 241-253.

D'Occhio MJ, Sudha G, Jillella D, Whyte T, Maclellan LJ, Walsh J, Trigg TE & Miller D 1997b Use of a GnRH agonist to prevent the endogenous LH surge and injection of exogenous LH to induce ovulation in heifers superstimulated with FSH: a new model for superovulation. *Theriogenology* **47** 601-613.

Dadarwal D. 2012. Factors affecting maturation of bovine oocytes; hormonal environment, follicular and maternal aging. In *Veterinary Biomedical Science*, pp. 195.

Danilovich N, Babu PS, Xing W, Gerdes M, Krishnamurthy H & Sairam MR 2000 Estrogen deficiency, obesity, and skeletal abnormalities in follicle-stimulating hormone receptor knockout (FORKO) female mice. *Endocrinology* **141** 4295-4308.

Davis BJ, Lennard DE, Lee CA, Tiano HF, Morham SG, Wetsel WC & Langenbach R 1999 Anovulation in cyclooxygenase-2-deficient mice is restored by prostaglandin E2 and interleukin-1beta. *Endocrinology* **140** 2685-2695.

De La Fuente R & Eppig JJ 2001 Transcriptional activity of the mouse oocyte genome: companion granulosa cells modulate transcription and chromatin remodeling. *Developmental biology* **229** 224-236.

de la Sota RL, Simmen FA, Diaz T & Thatcher WW 1996 Insulin-like growth factor system in bovine first-wave dominant and subordinate follicles. *Biology of reproduction* **55** 803-812.

Delidow BC, Lynch JP, White BA & Peluso JJ 1992 Regulation of proto-oncogene expression and deoxyribonucleic acid synthesis in granulosa cells of perifused immature rat ovaries. *Biology of reproduction* **47** 428-435.

Delidow BC, White BA & Peluso JJ 1990 Gonadotropin induction of c-fos and c-myc expression and deoxyribonucleic acid synthesis in rat granulosa cells. *Endocrinology* **126** 2302-2306.

Desaulniers DM, Lussier JG, Goff AK, Bousquet D & Guilbault LA 1995 Follicular development and reproductive endocrinology during and after superovulation in heifers and mature cows displaying contrasting superovulatory responses. *Theriogenology* **44** 479-497.

Dias FC, Costa E, Adams GP, Mapletoft RJ, Kastelic JP, Dochi O & J S 2012a Effect of duration of growing phase of ovulatory follicles on oocyte competence in superstimulated cattle. *Reproduction, Fertility and Development*
.

Dias FC, Mapletoft RJ, Kastelic JP, Adams GP, Colazo MG, Stover BC, Dochi O & Singh J 2012b Effect of length of progesterone exposure during ovulatory wave development on pregnancy rate. *Theriogenology* **77** 437-444.

Diouf MN, Sayasith K, Lefebvre R, Silversides DW, Sirois J & Lussier JG 2006 Expression of phospholipase A2 group IVA (PLA2G4A) is upregulated by human chorionic gonadotropin in bovine granulosa cells of ovulatory follicles. *Biology of reproduction* **74** 1096-1103.

Downs SM, Mosey JL & Klinger J 2009 Fatty acid oxidation and meiotic resumption in mouse oocytes. *Molecular reproduction and development* **76** 844-853.

Dreyfus M, Dardik R, Suh BS, Amsterdam A & Lahav J 1992 Differentiation-controlled synthesis and binding of thrombospondin to granulosa cells. *Endocrinology* **130** 2565-2570.

Duffy DM, Seachord CL & Dozier BL 2005 An ovulatory gonadotropin stimulus increases cytosolic phospholipase A2 expression and activity in granulosa cells of primate periovulatory follicles. *The Journal of clinical endocrinology and metabolism* **90** 5858-5865.

Dunning KR, Cashman K, Russell DL, Thompson JG, Norman RJ & Robker RL 2010 Beta-oxidation is essential for mouse oocyte developmental competence and early embryo development. *Biology of reproduction* **83** 909-918.

Eppig JJ 1976 Analysis of mouse oogenesis in vitro. Oocyte isolation and the utilization of exogenous energy sources by growing oocytes. *The Journal of experimental zoology* **198** 375-382.

Eppig JJ 1996 Coordination of nuclear and cytoplasmic oocyte maturation in eutherian mammals. *Reproduction, fertility, and development* **8** 485-489.

Evers BM, Wang X, Zhou Z, Townsend CM, Jr., McNeil GP & Dobner PR 1995 Characterization of promoter elements required for cell-specific expression of the neurotensin/neuromedin N gene in a human endocrine cell line. *Molecular and cellular biology* **15** 3870-3881.

Fair T, Hyttel P & Greve T 1995 Bovine oocyte diameter in relation to maturational competence and transcriptional activity. *Molecular reproduction and development* **42** 437-442.

Fauque P, Jouannet P, Lesaffre C, Ripoche MA, Dandolo L, Vaiman D & Jammes H 2007 Assisted Reproductive Technology affects developmental kinetics, H19 Imprinting Control Region methylation and H19 gene expression in individual mouse embryos. *BMC developmental biology* **7** 116.

Fayad T, Lefebvre R, Nimpf J, Silversides DW & Lussier JG 2007 Low-density lipoprotein receptor-related protein 8 (LRP8) is upregulated in granulosa cells of bovine dominant follicle: molecular characterization and spatio-temporal expression studies. *Biology of reproduction* **76** 466-475.

Ferreira EM, Vireque AA, Adona PR, Meirelles FV, Ferriani RA & Navarro PA 2009 Cytoplasmic maturation of bovine oocytes: structural and biochemical modifications and acquisition of developmental competence. *Theriogenology* **71** 836-848.

Forabosco A, Sforza C, De Pol A, Vizzotto L, Marzona L & Ferrario VF 1991 Morphometric study of the human neonatal ovary. *The Anatomical record* **231** 201-208.

Fortune JE, Rivera GM & Yang MY 2004 Follicular development: the role of the follicular microenvironment in selection of the dominant follicle. *Animal reproduction science* **82-83** 109-126.

Franchimont P, Demoulin A & Valcke JC 1988 Endocrine, paracrine and autocrine control of follicular development. *Hormone and metabolic research = Hormon- und Stoffwechselforschung = Hormones et metabolisme* **20** 193-203.

Fuhrer F, Mayr B, Schellander K, Kalat M & Schleger W 1989 Maturation competence and chromatin behaviour in growing and fully grown cattle oocytes. *Zentralblatt fur Veterinarmedizin. Reihe A* **36** 285-291.

Galway AB, Lapolt PS, Tsafriri A, Dargan CM, Boime I & Hsueh AJ 1990 Recombinant follicle-stimulating hormone induces ovulation and tissue plasminogen activator expression in hypophysectomized rats. *Endocrinology* **127** 3023-3028.

Gao X, Greenbaum LM, Mahesh VB & Brann DW 1992 Characterization of the kinin system in the ovary during ovulation in the rat. *Biology of reproduction* **47** 945-951.

Garcia Guerra A, Tribulo A, Yapura J, J S & Mapletoft RJ 2012 Effect of length of superstimulatory treatment protocol on ovarian response and number of transferable embryos in beef cattle. *Theriogenology* **78** 353-360.

Garside SA, Harlow CR, Hillier SG, Fraser HM & Thomas FH 2010a Thrombospondin-1 inhibits angiogenesis and promotes follicular atresia in a novel in vitro angiogenesis assay. *Endocrinology* **151** 1280-1289.

Garside SA, Henkin J, Morris KD, Norvell SM, Thomas FH & Fraser HM 2010b A thrombospondin-mimetic peptide, ABT-898, suppresses angiogenesis and promotes follicular atresia in pre- and early-antral follicles in vivo. *Endocrinology* **151** 5905-5915.

Gibbons JR, Wiltbank MC & Ginther OJ 1997 Functional interrelationships between follicles greater than 4 mm and the follicle-stimulating hormone surge in heifers. *Biology of reproduction* **57** 1066-1073.

Gilbert I, Robert C, Dieleman S, Blondin P & Sirard MA 2011 Transcriptional effect of the LH surge in bovine granulosa cells during the peri-ovulation period. *Reproduction* **141** 193-205.

Gilbert I, Robert C, Vigneault C, Blondin P & Sirard MA 2012 Impact of the LH surge on granulosa cell transcript levels as markers of oocyte developmental competence in cattle. *Reproduction* **143** 735-747.

Ginther OJ, Beg MA, Bergfelt DR, Donadeu FX & Kot K 2001 Follicle selection in monovular species. *Biology of reproduction* **65** 638-647.

Ginther OJ, Bergfelt DR, Beg MA, Meira C & Kot K 2004 In vivo effects of an intrafollicular injection of insulin-like growth factor 1 on the mechanism of follicle deviation in heifers and mares. *Biology of reproduction* **70** 99-105.

Ginther OJ, Bergfelt DR, Kulick LJ & Kot K 1998 Pulsatility of systemic FSH and LH concentrations during follicular-wave development in cattle. *Theriogenology* **50** 507-519.

Ginther OJ, Bergfelt DR, Kulick LJ & Kot K 1999 Selection of the dominant follicle in cattle: establishment of follicle deviation in less than 8 hours through depression of FSH concentrations. *Theriogenology* **52** 1079-1093.

Ginther OJ, Bergfelt DR, Kulick LJ & Kot K 2000 Selection of the dominant follicle in cattle: role of estradiol. *Biology of reproduction* **63** 383-389.

Ginther OJ, Kastelic JP & Knopf L 1989a Compositions and characteristics of follicular waves during the bovine estrous cycle. *Animal reproduction science* **20** 187-200.

Ginther OJ, Kastelic JP & Knopf L 1989b Intraovarian relationships among dominant and subordinate follicles and the corpus luteum in heifers. *Theriogenology* **32** 787-795.

Ginther OJ, Knopf L & Kastelic JP 1989c Temporal associations among ovarian events in cattle during oestrous cycles with two and three follicular waves. *Journal of reproduction and fertility* **87** 223-230.

Ginther OJ, Kot K, Kulick LJ & Wiltbank MC 1997 Emergence and deviation of follicles during the development of follicular waves in cattle. *Theriogenology* **48** 75-87.

Ginther OJ, Wiltbank MC, Fricke PM, Gibbons JR & Kot K 1996 Selection of the dominant follicle in cattle. *Biology of reproduction* **55** 1187-1194.

Glencross RG 1987 Effect of pulsatile infusion of gonadotropin-releasing hormone on plasma oestradiol-17 beta concentrations and follicular development during naturally and artificially maintained high levels of plasma progesterone in heifers. *The Journal of endocrinology* **112** 77-85.

Glister C, Tannetta DS, Groome NP & Knight PG 2001 Interactions between follicle-stimulating hormone and growth factors in modulating secretion of steroids and

inhibin-related peptides by nonluteinized bovine granulosa cells. *Biology of reproduction* **65** 1020-1028.

Goldman S, Dirnfeld M, Abramovici H & Kraiem Z 1997 Triiodothyronine and follicle-stimulating hormone, alone and additively together, stimulate production of the tissue inhibitor of metalloproteinases-1 in cultured human luteinized granulosa cells. *The Journal of clinical endocrinology and metabolism* **82** 1869-1873.

Greenaway J, Gentry PA, Feige JJ, LaMarre J & Petrik JJ 2005 Thrombospondin and vascular endothelial growth factor are cyclically expressed in an inverse pattern during bovine ovarian follicle development. *Biology of reproduction* **72** 1071-1078.

Greenaway J, Lawler J, Moorehead R, Bornstein P, Lamarre J & Petrik J 2007 Thrombospondin-1 inhibits VEGF levels in the ovary directly by binding and internalization via the low density lipoprotein receptor-related protein-1 (LRP-1). *Journal of cellular physiology* **210** 807-818.

Gudi T, Casteel DE, Vinson C, Boss GR & Pilz RB 2000 NO activation of fos promoter elements requires nuclear translocation of G-kinase I and CREB phosphorylation but is independent of MAP kinase activation. *Oncogene* **19** 6324-6333.

Haghighat N & Van Winkle LJ 1990 Developmental change in follicular cell-enhanced amino acid uptake into mouse oocytes that depends on intact gap junctions and transport system Gly. *The Journal of experimental zoology* **253** 71-82.

Hamel M, Dufort I, Robert C, Gravel C, Leveille MC, Leader A & Sirard MA 2008 Identification of differentially expressed markers in human follicular cells associated with competent oocytes. *Human reproduction* **23** 1118-1127.

Hammond JM, Baranao JL, Skaleris D, Knight AB, Romanus JA & Rechler MM 1985 Production of insulin-like growth factors by ovarian granulosa cells. *Endocrinology* **117** 2553-2555.

Harlow CR, Bradshaw AC, Rae MT, Shearer KD & Hillier SG 2007 Oestrogen formation and connective tissue growth factor expression in rat granulosa cells. *The Journal of endocrinology* **192** 41-52.

Harlow CR, Davidson L, Burns KH, Yan C, Matzuk MM & Hillier SG 2002 FSH and TGF-beta superfamily members regulate granulosa cell connective tissue growth factor gene expression in vitro and in vivo. *Endocrinology* **143** 3316-3325.

Harlow CR & Hillier SG 2002 Connective tissue growth factor in the ovarian paracrine system. *Molecular and cellular endocrinology* **187** 23-27.

Hayashi KG, Ushizawa K, Hosoe M & Takahashi T 2010 Differential genome-wide gene expression profiling of bovine largest and second-largest follicles: identification of genes associated with growth of dominant follicles. *Reproductive biology and endocrinology : RB&E* **8** 11.

Hazzard TM, Molskness TA, Chaffin CL & Stouffer RL 1999 Vascular endothelial growth factor (VEGF) and angiopoietin regulation by gonadotrophin and steroids in macaque granulosa cells during the peri-ovulatory interval. *Molecular human reproduction* **5** 1115-1121.

Hellberg P, Larson L, Olofsson J, Hedin L & Brannstrom M 1991 Stimulatory effects of bradykinin on the ovulatory process in the in vitro-perfused rat ovary. *Biology of reproduction* **44** 269-274.

Hernandez-Gonzalez I, Gonzalez-Robayna I, Shimada M, Wayne CM, Ochsner SA, White L & Richards JS 2006 Gene expression profiles of cumulus cell oocyte complexes during ovulation reveal cumulus cells express neuronal and immune-related genes: does this expand their role in the ovulation process? *Molecular endocrinology* **20** 1300-1321.

Hester KE, Harper MJ & Duffy DM 2010 Oral administration of the cyclooxygenase-2 (COX-2) inhibitor meloxicam blocks ovulation in non-human primates when administered to simulate emergency contraception. *Human reproduction* **25** 360-367.

Hou X, Arvisais EW, Jiang C, Chen DB, Roy SK, Pate JL, Hansen TR, Rueda BR & Davis JS 2008 Prostaglandin F2alpha stimulates the expression and secretion of transforming growth factor B1 via induction of the early growth response 1 gene (EGR1) in the bovine corpus luteum. *Molecular endocrinology* **22** 403-414.

Huang HC, Nguyen T & Pickett CB 2000 Regulation of the antioxidant response element by protein kinase C-mediated phosphorylation of NF-E2-related factor 2. *Proceedings of the National Academy of Sciences of the United States of America* **97** 12475-12480.

Hunzicker-Dunn M & Maizels ET 2006 FSH signaling pathways in immature granulosa cells that regulate target gene expression: branching out from protein kinase A. *Cellular signalling* **18** 1351-1359.

Hussein MR 2005 Apoptosis in the ovary: molecular mechanisms. *Human reproduction update* **11** 162-177.

Hussein MR, Haemel AK & Wood GS 2003a Apoptosis and melanoma: molecular mechanisms. *The Journal of pathology* **199** 275-288.

Hussein MR, Haemel AK & Wood GS 2003b p53-related pathways and the molecular pathogenesis of melanoma. *European journal of cancer prevention : the official journal of the European Cancer Prevention Organisation* **12** 93-100.

Hyttel P, Greve T & Callesen H 1989 Ultrastructural aspect of oocyte maturation and fertilization in cattle. *Journal Reproduction and Fertility Supplement* **38** 35-47.

Hyttel P, Xu KP, Smith S & Greve T 1986 Ultrastructure of in-vitro oocyte maturation in cattle. *Journal of reproduction and fertility* **78** 615-625.

Imai K, Khandoker MA, Yonai M, Takahashi T, Sato T, Ito A, Hasegawa Y & Hashizume K 2003 Matrix metalloproteinases-2 and -9 activities in bovine follicular fluid of different-sized follicles: relationship to intra-follicular inhibin and steroid concentrations. *Domestic animal endocrinology* **24** 171-183.

Ireland JJ 1987 Control of follicular growth and development. *Journal of reproduction and fertility. Supplement* **34** 39-54.

Jaiswal R.S., Singh J., Nagra H.S., Grafton T., Ratto M.H., Malhi P.S. & G.P. A 2006 Oocyte competence under different progestational environments. *Biology of reproduction* **Abstr#166.**

Jaiswal RS. 2007. Regulation of follicular wave pattern in cattle. In *Vetrinary Biomedical Science*.

Jaiswal RS, Singh J & Adams GP 2004 Developmental pattern of small antral follicles in the bovine ovary. *Biology of reproduction* **71** 1244-1251.

Jaiswal RS, Singh J, Marshall L & Adams GP 2009 Repeatability of 2-wave and 3-wave patterns of ovarian follicular development during the bovine estrous cycle. *Theriogenology* **72** 81-90.

Johnson ML, Murdoch J, Van Kirk EA, Kaltenbach JE & Murdoch WJ 1999 Tumor necrosis factor alpha regulates collagenolytic activity in preovulatory ovine follicles: relationship to cytokine secretion by the oocyte-cumulus cell complex. *Biology of reproduction* **61** 1581-1585.

Kallo I, Vida B, Deli L, Molnar CS, Hrabovszky E, Caraty A, Ciofi P, Coen CW & Liposits Z 2012 Co-localisation of kisspeptin with galanin or neurokinin B in afferents to mouse GnRH neurones. *Journal of neuroendocrinology* **24** 464-476.

Kerr B, Garcia-Rudaz C, Dorfman M, Paredes A & Ojeda SR 2009 NTRK1 and NTRK2 receptors facilitate follicle assembly and early follicular development in the mouse ovary. *Reproduction* **138** 131-140.

Kerr JF, Wyllie AH & Currie AR 1972 Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *British journal of cancer* **26** 239-257.

Kesler DJ & Garverick HA 1982 Ovarian cysts in dairy cattle: a review. *Journal of animal science* **55** 1147-1159.

Kinder JE, Garcia-Winder M, Imakawa K, Day ML, Zalesky DD, D'Occhio ML, Stumpf TT, Kittok RJ & Schanbacher BD 1991 Circulating concentrations of 17-estradiol influence pattern of LH in circulation of cows. *Domestic animal endocrinology* **8** 463-469.

Klipper E, Tatz E, Kisliouk T, Vlodavsky I, Moallem U, Schams D, Lavon Y, Wolfenson D & Meidan R 2009 Induction of heparanase in bovine granulosa cells by luteinizing hormone: possible role during the ovulatory process. *Endocrinology* **150** 413-421.

Knopf L, Kastelic JP, Schallenberger E & Ginther OJ 1989 Ovarian follicular dynamics in heifers: test of two-wave hypothesis by ultrasonically monitoring individual follicles. *Domestic animal endocrinology* **6** 111-119.

Kulick LJ, Kot K, Wiltbank MC & Ginther OJ 1999 Follicular and hormonal dynamics during the first follicular wave in heifers. *Theriogenology* **52** 913-921.

Kumar TR, Wang Y, Lu N & Matzuk MM 1997 Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility. *Nature genetics* **15** 201-204.

Kuno K, Terashima Y & Matsushima K 1999 ADAMTS-1 is an active metalloproteinase associated with the extracellular matrix. *The Journal of biological chemistry* **274** 18821-18826.

Kurusu S, Jinno M, Ehara H, Yonezawa T & Kawaminami M 2009 Inhibition of ovulation by a lipoxygenase inhibitor involves reduced cyclooxygenase-2 expression and prostaglandin E2 production in gonadotropin-primed immature rats. *Reproduction* **137** 59-66.

Laitinen M, Ristimaki A, Honkasalo M, Narko K, Paavonen K & Ritvos O 1997 Differential hormonal regulation of vascular endothelial growth factors VEGF, VEGF-B, and VEGF-C messenger ribonucleic acid levels in cultured human granulosa-luteal cells. *Endocrinology* **138** 4748-4756.

Laster DB 1972 Disappearance and uptake of (125 I)FSH in the rat, rabbit, ewe and cow. *Journal of reproduction and fertility* **30** 407-415.

Lee CN, Cook DL, Parfet JR, Smith CA, Youngquist RS & Garverick HA 1988 Induction of persistent ovarian follicular structures following administration of progesterone near the onset of estrus in dairy cattle. *Journal of dairy science* **71** 3505-3508.

Leonardsson G, Peng XR, Liu K, Nordstrom L, Carmeliet P, Mulligan R, Collen D & Ny T 1995 Ovulation efficiency is reduced in mice that lack plasminogen activator gene function: functional redundancy among physiological plasminogen activators. *Proceedings of the National Academy of Sciences of the United States of America* **92** 12446-12450.

Li F & Curry TE, Jr. 2009 Regulation and function of tissue inhibitor of metalloproteinase (TIMP) 1 and TIMP3 in periovulatory rat granulosa cells. *Endocrinology* **150** 3903-3912.

Li SS, Bigler J, Lampe JW, Potter JD & Feng Z 2005 FDR-controlling testing procedures and sample size determination for microarrays. *Statistics in medicine* **24** 2267-2280.

Liu J, Carriere PD, Dore M & Sirois J 1997 Prostaglandin G/H synthase-2 is expressed in bovine preovulatory follicles after the endogenous surge of luteinizing hormone. *Biology of reproduction* **57** 1524-1531.

Liu J, Kosma VM, Vanttinen T, Hyden-Granskog C & Voutilainen R 2002 Gonadotrophins inhibit the expression of insulin-like growth factor binding protein-related protein-2 mRNA in cultured human granulosa-luteal cells. *Molecular human reproduction* **8** 136-141.

Liu X, Wu H, Byrne M, Jeffrey J, Krane S & Jaenisch R 1995 A targeted mutation at the known collagenase cleavage site in mouse type I collagen impairs tissue remodeling. *The Journal of cell biology* **130** 227-237.

Liu Y, Sui HS, Wang HL, Yuan JH, Luo MJ, Xia P & Tan JH 2006 Germinal vesicle chromatin configurations of bovine oocytes. *Microscopy research and technique* **69** 799-807.

Liu Z, Youngquist RS, Garverick HA & Antoniou E 2009 Molecular mechanisms regulating bovine ovarian follicular selection. *Molecular reproduction and development* **76** 351-366.

Looney CR. 1986. Superovulation in beef females. . In *5th Annual Convention of AETA*, pp. 16-29.

Looney CR, Bondioli KR, Hill KG & Massey JM 1988 Superovulation of donor cows with bovine follicle-stimulating hormone (bFSH) produced by recombinant DNA technology. *Theriogenology* **29** 271 (Abstract).

Lopez-Romero P, Gonzalez MA, Callejas S, Dopazo A & Irizarry RA 2010 Processing of Agilent microRNA array data. *BMC research notes* **3** 18.

Loret de Mola JR, Goldfarb JM, Hecht BR, Baumgardner GP, Babbo CJ & Friedlander MA 1998 Gonadotropins induce the release of interleukin-1 beta, interleukin-6 and tumor necrosis factor-alpha from the human preovulatory follicle. *American journal of reproductive immunology* **39** 387-390.

Luo W, Gumen A, Haughian JM & Wiltbank MC 2011 The role of luteinizing hormone in regulating gene expression during selection of a dominant follicle in cattle. *Biology of reproduction* **84** 369-378.

Lydon JP, DeMayo FJ, Funk CR, Mani SK, Hughes AR, Montgomery CA, Jr., Shyamala G, Conneely OM & O'Malley BW 1995 Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. *Genes & development* **9** 2266-2278.

Madan P & Bridges PJ 2003 Expression of messenger RNA for ADAMTS subtypes changes in the periovulatory follicle after the gonadotropin surge and during luteal development and regression in cattle. *Biology of reproduction* **69** 1506-1514.

Mapletoft RJ & Bo GA 2011 The evolution of improved and simplified superovulation protocols in cattle. *Reproduction, fertility, and development* **24** 278-283.

Mapletoft RJ, Steward KB & Adams GP 2002 Recent advances in the superovulation in cattle. *Reproduction, nutrition, development* **42** 601-611.

Martinez MF, Kastelic JP, Adams GP, Janzen E, McCartney DH & Mapletoft RJ 2000 Estrus synchronization and pregnancy rates in beef cattle given CIDR-B, prostaglandin and estradiol, or GnRH. *The Canadian veterinary journal. La revue veterinaire canadienne* **41** 786-790.

McGray AJ, Gingerich T, Petrik JJ & LaMarre J 2011 Rapid insulin-like growth factor-1-induced changes in granulosa cell thrombospondin-1 expression in vitro. *The Journal of reproduction and development* **57** 76-83.

Mihm M & Austin EJ 2002 The final stages of dominant follicle selection in cattle. *Domestic animal endocrinology* **23** 155-166.

Mihm M, Baguisi A, Boland MP & Roche JF 1994 Association between the duration of dominance of the ovulatory follicle and pregnancy rate in beef heifers. *Journal of reproduction and fertility* **102** 123-130.

Mihm M, Baker PJ, Fleming LM, Monteiro AM & O'Shaughnessy PJ 2008 Differentiation of the bovine dominant follicle from the cohort upregulates mRNA expression for new tissue development genes. *Reproduction* **135** 253-265.

Mihm M, Baker PJ, Ireland JL, Smith GW, Coussens PM, Evans AC & Ireland JJ 2006 Molecular evidence that growth of dominant follicles involves a reduction in follicle-stimulating hormone dependence and an increase in luteinizing hormone dependence in cattle. *Biology of reproduction* **74** 1051-1059.

Mihm M, Crowe MA, Knight PG & Austin EJ 2002 Follicle wave growth in cattle. *Reproduction in domestic animals = Zuchthygiene* **37** 191-200.

Mihm M & Evans AC 2008 Mechanisms for dominant follicle selection in monovulatory species: a comparison of morphological, endocrine and intraovarian events in cows, mares and women. *Reproduction in domestic animals = Zuchthygiene* **43 Suppl 2** 48-56.

Monniaux D, Chupin D & Saumande J 1983 Superovulatory responses of cattle ovaries of cows. *Theriogenology* **19** 55-81.

Monteiro FM, Ferreira MM, Potiens JR, Eberhardt BG, Trinca LA & Barros CM 2010 Influence of superovulatory protocols on in vitro production of Nellore (Bos indicus) embryos. *Reproduction in domestic animals = Zuchthygiene* **45** 860-864.

Moor RM, Kruip TAM & Green D 1984 Intraovarian control of folliculogenesis: limits to superovulation? [Ovulation rate in domestic animals]. *Theriogenology* **21** 103-116.

Morham SG, Langenbach R, Loftin CD, Tiano HF, Vouloumanos N, Jennette JC, Mahler JF, Kluckman KD, Ledford A, Lee CA & Smithies O 1995 Prostaglandin synthase 2 gene disruption causes severe renal pathology in the mouse. *Cell* **83** 473-482.

Morrow DA 1969 Estrous behavior and ovarian activity in prepuberal and postpuberal dairy heifers. *Journal of dairy science* **52** 224-227.

Mundim TCD, Ramos AF, Sartori R, Dode MAN, Melo EO, Gomes LFS, Rumpf R & Franco MM 2009 Changes in gene expression profiles of bovine embryos produced in vitro, by natural ovulation, or hormonal superstimulation. *Genetics and Molecular Research* **8** 1398-1407.

Murdoch WJ, Colgin DC & Ellis JA 1997 Role of tumor necrosis factor-alpha in the ovulatory mechanism of ewes. *Journal of animal science* **75** 1601-1605.

Murdoch WJ & Gottsch ML 2003 Proteolytic mechanisms in the ovulatory folliculo-luteal transformation. *Connective tissue research* **44** 50-57.

Nasser LF, Adams GP, Bo GA & Mapletoft RJ 1993 Ovarian superstimulatory response relative to follicular wave emergence in heifers. *Theriogenology* **40** 713-724.

Ndiaye K, Fayad T, Silversides DW, Sirois J & Lussier JG 2005 Identification of downregulated messenger RNAs in bovine granulosa cells of dominant follicles following stimulation with human chorionic gonadotropin. *Biology of reproduction* **73** 324-333.

Nivet AL, Bunel A, Labrecque R, Belanger J, Vigneault C, Blondin P & Sirard MA 2012 FSH withdrawal improves developmental competence of oocytes in the bovine model. *Reproduction* **143** 165-171.

Noble KM, Tebble JE, Harvey D & Dobson H 2000 Ultrasonography and hormone profiles of persistent ovarian follicles (cysts) induced with low doses of progesterone in cattle. *Journal of reproduction and fertility* **120** 361-366.

Nogueira MF & Barros CM 2003 Time of ovulation in Nelore cows superstimulated with P36 protocol. *Acta Scientiae Veterinariae* **31**.

Nogueira MF, Buratini J, Jr., Price CA, Castilho AC, Pinto MG & Barros CM 2007 Expression of LH receptor mRNA splice variants in bovine granulosa cells: changes with follicle size and regulation by FSH in vitro. *Molecular reproduction and development* **74** 680-686.

Noseir WM 2003 Ovarian follicular activity and hormonal profile during estrous cycle in cows: the development of 2 versus 3 waves. *Reproductive biology and endocrinology : RB&E* **1** 50.

Pall M, Friden BE & Brannstrom M 2001 Induction of delayed follicular rupture in the human by the selective COX-2 inhibitor rofecoxib: a randomized double-blind study. *Human reproduction* **16** 1323-1328.

Park JY, Su YQ, Ariga M, Law E, Jin SL & Conti M 2004 EGF-like growth factors as mediators of LH action in the ovulatory follicle. *Science* **303** 682-684.

Parrish JJ, Krogenaes A & Susko-Parrish JL 1995 Effect of bovine sperm separation by either swim-up or Percoll method on success of in vitro fertilization and early embryonic development. *Theriogenology* **44** 859-869.

Peluffo MC, Murphy MJ, Baughman ST, Stouffer RL & Hennebold JD 2011 Systematic analysis of protease gene expression in the rhesus macaque ovulatory follicle: metalloproteinase involvement in follicle rupture. *Endocrinology* **152** 3963-3974.

Pierson A & Ginther OJ 1988 Ultrasonic imaging of the ovaries and uterus in cattle. *Theriogenology* **29** 21-37.

Pierson RA & Ginther OJ 1984 Ultrasonography of the bovine ovary. *Theriogenology* **21** 495-504.

Pierson RA & Ginther OJ 1986 Ovarian follicular populations during early pregnancy in heifers. *Theriogenology* **26** 649-659.

Pierson RA & Ginther OJ 1987 Follicular populations during the estrous cycle in heifers: Influence of day. *Animal reproduction science* **14** 165-176.

Prentice JR, Singh J, Dochi O & Anzar M 2011 Factors affecting nuclear maturation, cleavage and embryo development of vitrified bovine cumulus-oocyte complexes. *Theriogenology* **75** 602-609.

Rajakoski E 1960 The ovarian follicular system in sexually mature heifers with special reference to seasonal, cyclical, and left-right variations. *Acta Endocrinology* **34** 7-68.

Rathbone MJ, Kinder JE, Fike K, Kojima F, Clopton D, Ogle CR & Bunt CR 2001 Recent advances in bovine reproductive endocrinology and physiology and their impact on drug delivery system design for the control of the estrous cycle in cattle. *Advanced drug delivery reviews* **50** 277-320.

Reames PS, Hatler TB, Hayes SH, Ray DL & Silvia WJ 2011 Differential regulation of estrous behavior and luteinizing hormone secretion by estradiol-17beta in ovariectomized dairy cows. *Theriogenology* **75** 233-240.

Reisinger K, Baal N, McKinnon T, Munstedt K & Zygmunt M 2007 The gonadotropins: tissue-specific angiogenic factors? *Molecular and cellular endocrinology* **269** 65-80.

Revah I & Butler WR 1996 Prolonged dominance of follicles and reduced viability of bovine oocytes. *Journal of reproduction and fertility* **106** 39-47.

Rice VM, Limback SD, Roby KF & Terranova PF 1998 Changes in circulating and ovarian concentrations of bioactive tumour necrosis factor alpha during the first ovulation at puberty in rats and in gonadotrophin-treated immature rats. *Journal of reproduction and fertility* **113** 337-341.

Richards JS 1994 Hormonal control of gene expression in the ovary. *Endocrine reviews* **15** 725-751.

Richards JS, Russell DL, Robker RL, Dajee M & Alliston TN 1998 Molecular mechanisms of ovulation and luteinization. *Molecular and cellular endocrinology* **145** 47-54.

Rivera GM & Fortune JE 2003 Proteolysis of insulin-like growth factor binding proteins -4 and -5 in bovine follicular fluid: implications for ovarian follicular selection and dominance. *Endocrinology* **144** 2977-2987.

Roberge S, Rieger D & Rawlings NC 1995 Periovulatory LH, FSH and steroid hormone profiles in superovulated and unstimulated Holstein heifers. *Theriogenology* **44** 59-70.

Robker RL & Richards JS 1998a Hormonal control of the cell cycle in ovarian cells: proliferation versus differentiation. *Biology of reproduction* **59** 476-482.

Robker RL & Richards JS 1998b Hormone-induced proliferation and differentiation of granulosa cells: a coordinated balance of the cell cycle regulators cyclin D2 and p27Kip1. *Molecular endocrinology* **12** 924-940.

Rossignol S, Steunou V, Chalas C, Kerjean A, Rigolet M, Viegas-Pequignot E, Jouannet P, Le Bouc Y & Gicquel C 2006 The epigenetic imprinting defect of patients with Beckwith-Wiedemann syndrome born after assisted reproductive technology is not restricted to the 11p15 region. *Journal of medical genetics* **43** 902-907.

Rusovici R & LaVoie HA 2003 Expression and distribution of AP-1 transcription factors in the porcine ovary. *Biology of reproduction* **69** 64-74.

Russell DL & Robker RL 2007 Molecular mechanisms of ovulation: co-ordination through the cumulus complex. *Human reproduction update* **13** 289-312.

Sakata M, Kobayashi H, Sun GW, Mochizuki O, Takagi A & Kojima T 2000 Ryudocan expression by luteinized granulosa cells is associated with the process of follicle atresia. *Fertility and sterility* **74** 1208-1214.

Salamone DF, Adams GP & Mapletoft RJ 1999 Changes in the cumulus-oocyte complex of subordinate follicles relative to follicular wave status in cattle. *Theriogenology* **52** 549-561.

Sarvari M, Kallo I, Hrabovszky E, Solymosi N, Toth K, Liko I, Molnar B, Tihanyi K & Liposits Z 2010 Estradiol replacement alters expression of genes related to neurotransmission and immune surveillance in the frontal cortex of middle-aged, ovariectomized rats. *Endocrinology* **151** 3847-3862.

Sasson R, Rimon E, Dantes A, Cohen T, Shinder V, Land-Bracha A & Amsterdam A 2004 Gonadotrophin-induced gene regulation in human granulosa cells obtained from IVF patients. Modulation of steroidogenic genes, cytoskeletal genes and genes coding for apoptotic signalling and protein kinases. *Molecular human reproduction* **10** 299-311.

Sato A, Otsu E, Negishi H, Utsunomiya T & Arima T 2007 Aberrant DNA methylation of imprinted loci in superovulated oocytes. *Human reproduction* **22** 26-35.

Savio JD, Keenan L, Boland MP & Roche JF 1988 Pattern of growth of dominant follicles during the oestrous cycle of heifers. *Journal of reproduction and fertility* **83** 663-671.

Savio JD, Thatcher WW, Morris GR, Entwistle K, Drost M & Mattiacci MR 1993 Effects of induction of low plasma progesterone concentrations with a progesterone-releasing intravaginal device on follicular turnover and fertility in cattle. *Journal of reproduction and fertility* **98** 77-84.

Sayasith K, Brown KA, Lussier JG, Dore M & Sirois J 2006 Characterization of bovine early growth response factor-1 and its gonadotropin-dependent regulation in ovarian follicles prior to ovulation. *Journal of molecular endocrinology* **37** 239-250.

Scanlon PF, Sreenan J & Gordon I 1968 Hormonal induction of superovulation in cattle. *Journal Agricuture Science* **70** 179-182.

Schams D, Kosmann M, Berisha B, Amselgruber WM & Miyamoto A 2001 Stimulatory and synergistic effects of luteinising hormone and insulin like growth factor 1 on the secretion of vascular endothelial growth factor and progesterone of cultured bovine granulosa cells. *Experimental and clinical endocrinology & diabetes : official journal, German Society of Endocrinology [and] German Diabetes Association* **109** 155-162.

Schindler R, Nilsson E & Skinner MK 2010 Induction of ovarian primordial follicle assembly by connective tissue growth factor CTGF. *PloS one* **5** e12979.

Senger PL 2005 *Pathways to pregnancy and parturition*: Current Conceptions, Inc.

Sharma SC & Richards JS 2000 Regulation of AP1 (Jun/Fos) factor expression and activation in ovarian granulosa cells. Relation of JunD and Fra2 to terminal differentiation. *The Journal of biological chemistry* **275** 33718-33728.

Shimada M, Hernandez-Gonzalez I, Gonzalez-Robayna I & Richards JS 2006 Paracrine and autocrine regulation of epidermal growth factor-like factors in cumulus oocyte complexes and granulosa cells: key roles for prostaglandin synthase 2 and progesterone receptor. *Molecular endocrinology* **20** 1352-1365.

Shimizu T, Jayawardana BC, Tetsuka M & Miyamoto A 2007 Differential effect of follicle-stimulating hormone and estradiol on expressions of vascular endothelial growth factor (VEGF) 120, VEGF164 and their receptors in bovine granulosa cells. *The Journal of reproduction and development* **53** 105-112.

Shindo T, Kurihara H, Kuno K, Yokoyama H, Wada T, Kurihara Y, Imai T, Wang Y, Ogata M, Nishimatsu H, Moriyama N, Oh-hashi Y, Morita H, Ishikawa T, Nagai R, Yazaki Y & Matsushima K 2000 ADAMTS-1: a metalloproteinase-disintegrin essential for normal growth, fertility, and organ morphology and function. *The Journal of clinical investigation* **105** 1345-1352.

Shozu M, Minami N, Yokoyama H, Inoue M, Kurihara H, Matsushima K & Kuno K 2005 ADAMTS-1 is involved in normal follicular development, ovulatory process and organization of the medullary vascular network in the ovary. *Journal of molecular endocrinology* **35** 343-355.

Silva JM, Hamel M, Sahmi M & Price CA 2006 Control of oestradiol secretion and of cytochrome P450 aromatase messenger ribonucleic acid accumulation by FSH involves different intracellular pathways in oestrogenic bovine granulosa cells in vitro. *Reproduction* **132** 909-917.

Singh J, Dominguez M, Jaiswal R & Adams GP 2004 A simple ultrasound test to predict the superstimulatory response in cattle. *Theriogenology* **62** 227-243.

Singh J, Pierson RA & Adams GP 1998 Ultrasound image attributes of bovine ovarian follicles and endocrine and functional correlates. *Journal of reproduction and fertility* **112** 19-29.

Sirard MA, Picard L, Dery M, Coenen K & Blondin P 1999 The time interval between FSH administration and ovarian aspiration influences the development of cattle oocytes. *Theriogenology* **51** 699-708.

Sirois J & Fortune JE 1988 Ovarian follicular dynamics during the estrous cycle in heifers monitored by real-time ultrasonography. *Biology of reproduction* **39** 308-317.

Sirois J & Richards JS 1993 Transcriptional regulation of the rat prostaglandin endoperoxide synthase 2 gene in granulosa cells. Evidence for the role of a cis-acting C/EBP beta promoter element. *The Journal of biological chemistry* **268** 21931-21938.

Sisco B, Hagemann LJ, Shelling AN & Pfeffer PL 2003 Isolation of genes differentially expressed in dominant and subordinate bovine follicles. *Endocrinology* **144** 3904-3913.

Sisco B & Pfeffer PL 2007 Expression of activin pathway genes in granulosa cells of dominant and subordinate bovine follicles. *Theriogenology* **68** 29-37.

Smith MJ & Jennes L 2001 Neural signals that regulate GnRH neurones directly during the oestrous cycle. *Reproduction* **122** 1-10.

Smith ML, Chen IT, Zhan Q, Bae I, Chen CY, Gilmer TM, Kastan MB, O'Connor PM & Fornace AJ, Jr. 1994 Interaction of the p53-regulated protein Gadd45 with proliferating cell nuclear antigen. *Science* **266** 1376-1380.

Smyth GK 2004 Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Statistical Applications in Genetics and Molecular Biology* **3** 3.

Smyth GK 2005. Limma: Linear Models for microarray data. In *Bioinformatics and Computational Biology Solutions using R and Bioconductor*, pp. 473.

Sterneck E, Tessarollo L & Johnson PF 1997 An essential role for C/EBPbeta in female reproduction. *Genes & development* **11** 2153-2162.

Stojkovic M, Machado SA, Stojkovic P, Zakhartchenko V, Hutzler P, Goncalves PB & Wolf E 2001 Mitochondrial distribution and adenosine triphosphate content of bovine oocytes before and after in vitro maturation: correlation with morphological criteria and developmental capacity after in vitro fertilization and culture. *Biology of reproduction* **64** 904-909.

Stringfellow DA & Seidel SM 1998 *'Manual of the International Embryo Transfer Society, 3rd edn.'* Savoy, IL, USA: International Embryo Transfer Society.

Takahashi T, Morrow JD, Wang H & Dey SK 2006 Cyclooxygenase-2-derived prostaglandin E(2) directs oocyte maturation by differentially influencing multiple signaling pathways. *The Journal of biological chemistry* **281** 37117-37129.

Thomas FH & Vanderhyden BC 2006 Oocyte-granulosa cell interactions during mouse follicular development: regulation of kit ligand expression and its role in oocyte growth. *Reproductive biology and endocrinology : RB&E* **4** 19.

Thomas FH, Wilson H, Silvestri A & Fraser HM 2008 Thrombospondin-1 expression is increased during follicular atresia in the primate ovary. *Endocrinology* **149** 185-192.

Todd DJ, Lee AH & Glimcher LH 2008 The endoplasmic reticulum stress response in immunity and autoimmunity. *Nature reviews. Immunology* **8** 663-674.

Tonhati H, Lobo RB & Oliveira HN 1999 Repeatability and heritability of response to superovulation in Holstein cows. *Theriogenology* **51** 1151-1156.

Tornell J, Billig H & Hillensjo T 1990 Resumption of rat oocyte meiosis is paralleled by a decrease in guanosine 3',5'-cyclic monophosphate (cGMP) and is inhibited by microinjection of cGMP. *Acta physiologica Scandinavica* **139** 511-517.

Townson DH & Combelles CMH 2012. Ovarian Follicular Atresia. In *Basic Gynecology - Some related issues*.

Townson DH, Tsang PC, Butler WR, Frajblat M, Griel LC, Jr., Johnson CJ, Milvae RA, Niksic GM & Pate JL 2002 Relationship of fertility to ovarian follicular waves before breeding in dairy cows. *Journal of animal science* **80** 1053-1058.

Tribulo A, Rogan D, Tribulo H, Tribulo R, Mapletoft RJ & Bo GA 2012 Superovulation of beef cattle with a split-single intramuscular administration of Folltropin-V in two concentrations of hyaluronan. *Theriogenology* **77** 1679-1685.

Urban RJ, Garmey JC, Shupnik MA & Veldhuis JD 1991 Follicle-stimulating hormone increases concentrations of messenger ribonucleic acid encoding cytochrome P450 cholesterol side-chain cleavage enzyme in primary cultures of porcine granulosa cells. *Endocrinology* **128** 2000-2007.

Vaknin KM, Lazar S, Popliker M & Tsafriri A 2001 Role of meiosis-activating sterols in rat oocyte maturation: effects of specific inhibitors and changes in the expression of lanosterol 14alpha-demethylase during the preovulatory period. *Biology of reproduction* **64** 299-309.

van den Hurk R & Zhao J 2005 Formation of mammalian oocytes and their growth, differentiation and maturation within ovarian follicles. *Theriogenology* **63** 1717-1751.

van Koppen CJ, Verbost PM, van de Lagemaat R, Karstens WJ, Loozen HJ, van Achterberg TA, van Amstel MG, Brands JH, van Doornmalen EJ, Wat J, Mulder SJ, Raafs BC, Verkaik S, Hanssen RG & Timmers CM 2013 Signaling of an allosteric, nanomolar potent, low molecular weight agonist for the follicle-stimulating hormone receptor. *Biochemical pharmacology* **85** 1162-1170.

van Montfoort AP, Geraedts JP, Dumoulin JC, Stassen AP, Evers JL & Ayoubi TA 2008 Differential gene expression in cumulus cells as a prognostic indicator of embryo viability: a microarray analysis. *Molecular human reproduction* **14** 157-168.

Varani S, Elvin JA, Yan C, DeMayo J, DeMayo FJ, Horton HF, Byrne MC & Matzuk MM 2002 Knockout of pentraxin 3, a downstream target of growth differentiation factor-9, causes female subfertility. *Molecular endocrinology* **16** 1154-1167.

Wandji SA, Gadsby JE, Barber JA & Hammond JM 2000 Messenger ribonucleic acids for MAC25 and connective tissue growth factor (CTGF) are inversely regulated during folliculogenesis and early luteogenesis. *Endocrinology* **141** 2648-2657.

Wildt DE, Woody HD & Dukelow WR 1975 Induction of multiple ovulation in the cow with single injections of FSH and HCG. *Journal of reproduction and fertility* **44** 583-586.

Willmott N, Saunders J, Bo GA, Palasz A, Pierson RA & Mapletoft RJ 1990 The effect of FSH/LH ratio in pituitary extracts on superovulatory response in the cow. . *Theriogenology* **33** 347 (Abstract).

Wu LL, Russell DL, Norman RJ & Robker RL 2012 Endoplasmic reticulum (ER) stress in cumulus-oocyte complexes impairs pentraxin-3 secretion, mitochondrial membrane potential (DeltaPsi m), and embryo development. *Molecular endocrinology* **26** 562-573.

Yancopoulos GD, Davis S, Gale NW, Rudge JS, Wiegand SJ & Holash J 2000 Vascular-specific growth factors and blood vessel formation. *Nature* **407** 242-248.

Yasuda K, Hagiwara E, Takeuchi A, Mukai C, Matsui C, Sakai A & Tamotsu S 2005 Changes in the distribution of tenascin and fibronectin in the mouse ovary during folliculogenesis, atresia, corpus luteum formation and luteolysis. *Zoological science* **22** 237-245.

Youngquist RS & Threlfall WR 2007 *Current Therapy in Large Animal Theriogenology*. Philadelphia: Elsevier.

Zalman Y, Klipper E, Farberov S, Mondal M, Wee G, Folger JK, Smith GW & Meidan R 2012 Regulation of angiogenesis-related prostaglandin f2alpha-induced genes in the bovine corpus luteum. *Biology of reproduction* **86** 92.

Zhang X, Jafari N, Barnes RB, Confino E, Milad M & Kazer RR 2005 Studies of gene expression in human cumulus cells indicate pentraxin 3 as a possible marker for oocyte quality. *Fertility and sterility* **83 Suppl 1** 1169-1179.

Zhou J, Kumar TR, Matzuk MM & Bondy C 1997 Insulin-like growth factor I regulates gonadotropin responsiveness in the murine ovary. *Molecular endocrinology* **11** 1924-1933.

Zimbelman RG & Smith LW 1966 Control of ovulation in cattle with melengestrol acetate. II. Effects on follicular size and activity. *Journal of reproduction and fertility* **11** 193-201.

Zolti M, Meirom R, Shemesh M, Wollach D, Mashiach S, Shore L & Rafael ZB 1990 Granulosa cells as a source and target organ for tumor necrosis factor-alpha. *FEBS letters* **261** 253-255.